# ENDOCRINE AND MOLECULAR REGULATION OF OVARIAN ANTRAL FOLLICULAR WAVE EMERGENCE AND GROWTH IN SHEEP

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## ABSTRACT

In sheep, large ovarian antral follicles grow in waves with a periodicity of every 4 to 5 days; each wave is initiated by a peak in serum concentrations of follicle stimulating hormone (FSH). In the present thesis, follicular data and hormone estimations acquired from daily ultrasonography and blood samples, respectively, were used to study mechanisms regulating the number of follicular waves per estrous cycle. Using additional approaches such as implants releasing estradiol-17ß and or progesterone, immunization against gonadotropin releasing hormone (GnRH), and injections of GnRH, the role of pulsed luteinizing hormone (LH) secretion and FSH peaks in follicular wave emergence and growth and the dependency of FSH peaks on pulsed GnRH secretion, were studied in sheep. The viability of aged follicles was also addressed.

The results of the present studies showed that ewes with three or four waves per cycle had cycles of the same length. The inter-wave interval was longer for the first and the last or ovulatory wave of the cycle in three compared to four wave cycles. The length of the lifespan and regression phase of the largest follicle of a wave declined across the cycle as FSH peak concentration and amplitude decreased. The maximum follicular diameter of the largest follicle growing in the first wave and the last or ovulatory wave of the cycle was greater compared to other waves of the cycle. Treatment of anestrous ewes with estradiol releasing implants alone completely abolished pulsed LH secretion and suppressed follicular wave development; however, FSH secretion was only minimally affected and the pool of small follicles was not affected. When pulsed secretion of LH was restored by frequent injections of GnRH, follicular waves were re-established. Treatment of anestrous ewes with implants releasing estradiol and progesterone, decreased FSH peak amplitude and abolished LH pulses and follicular waves; the size of the pool of small follicles increased. Immunization against GnRH in anestrous ewes abolished pulsatile LH secretion and suppressed follicular wave emergence; however, FSH peaks continued to occur for several weeks. In cyclic ewes, creating an LH pulse frequency typical of the follicular phase, during the luteal phase of the cycle by giving GnRH, increased maximum diameter of the largest follicle in a wave and serum concentrations of estradiol and progesterone. The enhanced growth of follicles in a wave blocked the next expected FSH peak and its associated follicular wave. Decreasing LH pulse frequencies lower than the minimal

frequency seen in the luteal phase, by implants releasing progesterone, did not affect the growth of follicular waves.

It was previously demonstrated that treatment of non-prolific WWF ewes with Prostaglandin $F_{2a}$ ($PGF_{2a}$) and medroxy progesterone acetate (MPA) increased the ovulation rate by adding ovulations from the penultimate wave in addition to the final wave of the cycle; however, fertility was not improved. In the last study of my thesis, we collected follicles, with an extended lifespan, from the penultimate wave of the cycle in ewes given the PGF2a and MPA treatment. We compared their quality with follicles from the final wave of the cycle by looking at the expression of markers of follicular development. The results showed that theca cells of follicles from the final wave had significantly higher mRNA expression for vascular endothelial growth factor (VEGF) compared to follicles from the penultimate wave. Granulosa cells of follicles from the final wave had significantly higher mRNA expression for connexion 43 (Cx43) compared to follicles from the penultimate wave. Protein expression for Cx43, proliferating cell nuclear antigen (PCNA) and Factor VIII was greater in follicles from the final compared to the penultimate wave.

We concluded from the present studies that: 1) the mechanism that makes a three wave or four wave cycle is unclear; 2) some level of pulsatile LH secretion is required for an FSH peak to trigger emergence of follicular waves in anestrous ewes; 3) progesterone enhances the inhibitory effects of estradiol on FSH secretion in anestrous ewes, suppressing specifically FSH peak amplitude; 4) an endogenous rhythm may exist that drives the peaks in FSH secretion independent of secretory products from the follicles growing in a wave and pulsed GnRH secretion; 5) follicular waves in ewes, when exposed to an LH pulse frequency similar to the follicular phase, during the luteal phase of the cycle, when serum progesterone concentrations are high, can grow and function like ovulatory follicles growing in the follicular phase of the cycle; 6) expression of some markers of vascularization/ angiogenesis, gap-junctional communication and cell proliferation, appeared to be decreased in follicles from the penultimate compared to the final wave of an estrous cycle, when the lifespan of follicles from the penultimate wave was extended such that they were present in the ovary with follicles from the final wave of the cycle.

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# *To My Parents*

***"For their Love, Support and Encouragement"***

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| | |
|---|---|
| ANOVA | Analysis of variance |
| cAMP | Cyclic adenosine monophosphate |
| CH | Corpus hemorrhagicum |
| CL | Corpus luteum |
| CV | Coefficient of variation |
| Cx | Connexin |
| d | days |
| dpm | disintegrations per minute |
| $E_2$ | Estradiol-17ß |
| EB | Estradiol benzoate |
| ER | Estradiol receptor |
| FGF-2 | Fibroblast growth factor-2 |
| FSH | Follicle stimulating hormone |
| FSHr | FSH receptor |
| FW | Final wave |
| GnRH | Gonadotropin releasing hormone |
| GnRHr | GnRH receptor |
| h | Hour/s |
| hCG | Human chorionic gonadotropin |
| HPO | Hypothalamic-pituitary-ovarian axis |
| i.d | inside diameter |
| i.m. | Intra-muscular |
| i.v | Intra-venous |
| IVF | Invitro fertilization |
| IWI | Inter-wave iterval |
| LF | Luteinized follicle |
| LH | Luteinizing hormone |
| LI | Labelling index |
| LHr | Luteinizing hormone receptor |

| | |
|---|---|
| LHRH | Luteinizing hormone releasing hormone |
| LSD | Least significant difference |
| MPA | Medroxyprogesterone acetate |
| mRNA | Messenger ribonucleic acid |
| NOS3 | Endothelial nitric oxide syntahse |
| o.d | outside diameter |
| oFSH | Ovine FSH |
| OL | Ovalbumin- Luteinizing hormone releasing hormone-7 |
| oLH | Ovine LH |
| OVX | Ovariectomized ewes |
| $P_4$ | Progesterone |
| PBS | Phosphate buffered saline |
| PCNA | Proliferating cell nuclear antigen |
| PDAR | Predetermined asssay reagent kit |
| PEG | Polyethylene glycol |
| $PGF_{2\forall}$ | Prostaglandin $F_{2\forall}$ |
| PR | Progesterone receptor |
| PW | Penultimate wave |
| RIA | Radioimmunoassay |
| RM ANOVA | Repeated measures analysis of variance |
| rRNA | Ribosomal RNA |
| S.E.M | Standard error of the mean |
| s.c. | Subcutaneous |
| TCM-199 | Tissue culture medium-199 |
| TGFß | Transforming growth factor-ß |
| TL | Thioredoxin-Luteinizing hormone releasing hormone-7 |
| VEGF | Vascular endothelial growth factor |
| WWF | Western White Face |
| ZP | Zona pellucida |

# Chapter 1: LITERATURE REVIEW

## 1.1 Introduction

The main focus of the research presented in this thesis is the follicle, which is one of the structural and functional units of the ovary. In recent years, the blend of ultrasonography and hormone assays along with molecular techniques has provided an excellent approach towards understanding the regulation of follicular development in many species. The studies described in this thesis were performed to explore the gonadotropic and steroidal regulation of follicular development in cyclic and anestrous ewes. Experiments were conducted to investigate the regulation of the number of follicular waves during an estrous cycle and the role of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in follicular wave emergence and growth. In addition, the quality of aged follicles was examined by investigating certain markers of follicular development. Such knowledge will enhance our existing understanding of the endocrine regulation of follicular development and further might enable us to improve methods to control ovarian function and thus reproduction in sheep *(Ovis aris)*.

Methods utilized in the studies described in this thesis were transrectal ultrasonography, radioimmunoassay (RIA), immunohistochemistry (IHC) and real-time polymerase chain reaction (RT-PCR). In this chapter, the focus is on literature concerning research findings in sheep but wherever important information appeared to be lacking for sheep, literature from other species, including humans, was incorporated.

## 1.2 The estrous cycle in the ewe

An estrous cycle is the period between one estrus and the next or the interval between two ovulations (Hafez, 1952). Estrus is the period of sexual receptivity; the period when females allow copulation. Sheep are considered *seasonally polyestrous* animals as most breeds exhibit estrous cycles only during certain seasons of the year (Hafez, 1952). Sheep are *short day breeders* as they show estrous cycles as the day length decreases i.e during fall and early winter (breeding season) (Hafez, 1952; Marshall, 1937). During the remainder of the year (spring and summer), the ewe generally fails to display estrous cycles and ovulations; this season is referred to as the non-breeding or anestrous season (Hafez, 1952; Marshall, 1937). Sheep are *spontaneous ovulators* as they do not require the act of copulation to induce ovulation (Hafez, 1952).

The length of the estrous cycle in sheep is 16-17 d (Marshall, 1904).This is fairly consistent among different breeds and does not vary with age (McKinszie and Terrill, 1937). Abnormally short or long ovarian cycles are sometimes observed in sheep. Long cycles may be associated with the extended lifespan of the corpus luteum (CL; O'Shea et al., 1986). Short cycles are observed throughout the post-partum period and shortly after the onset of the breeding season (Bartlewski et al., 2000b). Short cycles are usually associated with short-lived CL and inadequate luteinization (Hunter, 1991). Unlike the length of the estrous cycle, there is remarkable variation in the lengths of the breeding and anestrous seasons between different breeds of sheep (Goodman, 1994). Among the breeds located close to the equator (tropical regions), there is no particular annual rhythm of ovarian activity and ewes can breed throughout the year

(Ali et al., 2006). In contrast, those breeds located in regions of high latitude (temperate regions), begin to cycle in late summer and continue until late winter (Karsch et al., 1979).

### 1.2.1 The phases of the estrous cycle

The estrous cycle can be divided into 4 phases; proestrus, estrus, metestrus and diestrus (Goodman, 1994). Proestrus is the period preceding behavioral estrus. Proestrus is characterized by a decrease in progesterone secretion and an increase in estradiol secretion as a result of regression of the CL and recruitment and growth of large antral follicles for ovulation. Estrus is characterized by high serum concentrations of estradiol which, after a period of exposure to progesterone, lead to estrous behaviors such as increased locomotion, vocalizations, nervousness, rapid tail movement or raising of tail and standing for breeding in the presence of the ram. In ewes, behavioral estrus lasts for 1-2 d, depending on the breed (Bindon et al., 1979; Goodman, 1994; Quirke et al., 1979). Ovulation occurs at or near the end of behavioral estrus (24-30 h after the onset of estrus behavior; McKinszie and Terrill, 1937). Proestrus and estrus constitute the follicular phase of the estrous cycle.

Metestrus follows estrus and is characterized by growth of the CL and increasing serum progesterone concentrations (Keyes et al., 1983). The growing CL at this stage is referred to as a corpus haemorrhagicum (CH). Diestrus is the longest period of the estrous cycle and is characterized by formation of a fully functional CL from the CH and maximum serum concentrations of progesterone. Metestrus and diestrus constitute the luteal phase of the estrous cycle.

## 1.3. Ovine estrous cycle endocrine profiles

Physiologically, estrous cycles are made possible by interactions among four important organs in the body; the hypothalamus, pituitary, ovaries and the uterus. The main hormones that are involved in the regulation of estrous cycle are: Gonadotropin releasing hormone (GnRH) from the hypothalamus; luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary; estradiol and inhibins from growing follicles; progesterone and oxytocin from the CL; and prostaglandin F2 alpha ($PGF_{2a}$) from the endometrium of the uterus (Scaramuzzi et al., 1993). Regulation of the hypothalamic-pituitary-ovarian (HPO) axis is mainly through control of GnRH secretion by ovarian steroids. Other factors such as the regulation of local blood supply, variation in the isoforms of LH and FSH that are secreted, changes in sensitivity of the ovaries and pituitary to gonadotropins and GnRH respectively are also involved in the control of the HPO axis. Changes in countercurrent exchange of hormones between blood and lymphatic vessels also play a role in regulation of the HPO axis (Koziorowski and Stefanezyk-Krzymowsha, 2007).

### 1.3.1. LH and FSH

In ewes, gonadotropins are released from the pituitary in two distinct patterns (Dyer, 1985); phasic and tonic. The Phasic or surge pattern of LH and FSH secretion consists of a massive release of LH and FSH which occurs approximately 14 h before ovulation (Figure 1.1, 1.2; Rawlings and Cook, 1993). This mode of secretion is also referred to as the preovulatory gonadotropin surge and phasic release of LH causes ovulation (Figure 1.2). Phasic secretion of gonadotropins lasts for 8-12 h (Bolt et al., 1971; Moenter et al., 1990) and is primarily induced by decreased progesterone and

increased estradiol secretions during the follicular phase of the estrous cycle (Baird and Scaramuzzi, 1976; Bolt et al., 1971; Jeffcoate et al., 1984; Joseph et al., 1992; Karsch et al., 1980; Moenter et al., 1990; Rawlings et al., 1984; Scaramuzzi et al., 1970). Tonic, episodic or pulsatile secertion of LH is seen throughout the estrous cycle and is generated in response to pulsatile secretion of GnRH from the hypothalamus (Levine et al., 1982; Rawlings and Cook, 1993). Pulsatile secretion of LH is also seen in ovariectomized ewes where, with the absence of ovarian estradiol and progesterone, pulse frequency and amplitude are very high (Gay and Sheath, 1972). Low amplitude LH pulses, secreted at a low frequency (one pulse every 3-4 h), occur during the mid-luteal phase of the estrous cycle (Figure 1.2; Bartlewski et al., 2000c; Goodman et al., 1981a). As the ewe transitions from the luteal to the follicular phase of the cycle, LH pulse frequency increases with a small decrease in pulse amplitude (Hauger et al., 1977; Jackson and Davis, 1979; Wheaton, 1979). LH pulses of high amplitude and frequency (one pulse every 20-30 min) are seen during the preovulatory LH surge (Figure 1.2; Baird, 1978; Goodman and Karsch, 1981; Rawlings and Cook, 1993). It has been suggested that the LH pulses are of lower amplitude on the upslope than on the downslope of the preovulatory surge (Rawlings and Cook, 1993). There is also an increase in the basal (non-pulsatile or serum LH concentrations upon elimination of pulses) secretion of LH during the preovulatory LH surge (Rawlings and Cook, 1993). In metestrus and diestrus, LH pulse frequency decreases and amplitude increases (Baird et al., 1976; Hauger et al., 1977). Pulsed secretion of LH is suppressed during anestrus and the number of LH pulses increase from 2-3 pulses per 9 h to 6 pulses per 9 h during transition from anestrous to the breeding season (McNatty et al., 1984b). Mean serum

LH concentrations are lower at the beginning compared to the mid and later periods of the breeding season (Malpaux et al., 1989; Malpaux et al., 1988; Rawlings et al., 1977).

At the time of estrus, there are two surges in FSH secertion. The first FSH surge is the preovulatory FSH surge and accompanies the preovulatory LH surge. The second FSH surge occurs 20-36 h later (Bister and Paquay, 1983; Findlay et al., 1990; Pant, 1977; Wheaton et al., 1984). The preovulatory FSH surge is of higher amplitude but shorter duration (11-12 h *versus* 20-24 h) compared to the second FSH surge (Baird et al., 1991; Wheaton et al., 1984). In earlier studies in intact ewes and based on observations from the peripheral circulation, FSH secretory profiles were seen to be non-pulsatile with only basal release (Bister and Paquay, 1983; Wallace and McNeilly, 1986; Wheaton et al., 1984). However, in overiectomized ewes, apart from pulses of FSH secretion in hypophyseal portal circulation (Padmanabhan et al., 2003; Padmanabhan et al., 1997; Padmanabhan and Sharma, 2001), there are reports suggesting pulses of FSH secretion in peripheral circulation (Padmanabhan et al., 1997). Some researchers have suggested that there are pulses of FSH secretion in the hypophyseal portal circulation during the estrous cycle in sheep (Padmanabhan et al., 2003; Padmanabhan and McNeilly, 2001). Others have shown that there are periodic peaks in serum FSH concentrations which occur every 4-5 d throughout the estrous cycle (Figure 1.1; Baird and McNeilly, 1981; Bartlewski et al., 1999a; Duggavathi et al., 2004; Evans et al., 2002; Souza et al., 1998). These peaks have a duration of 3 to 4 d (Bartlewski et al., 1999a; Duggavathi et al., 2005a). Periodic peaks in serum FSH concentrations are seen in anestrous and pregnant ewes and also in overiectomized ewes (Bartlewski et al., 1998; Bister and Paquay, 1983; Duggavathi et al., 2005a; Duggavathi

et al., 2004). These peaks in serum FSH concentrations are associated with emergence of follicular waves (Figure 1.1; Bartlewski et al., 1999a; Bartlewski et al., 1998; Bartlewski et al., 1999b; Evans et al., 2000; Ginther et al., 1995; Souza et al., 1998). The second FSH surge around estrus, mentioned above constitutes the FSH secretory peak occurring prior to emergence of the first follicular wave of the cycle in the ewe (Fortune et al., 1991). The amplitude and duration of FSH peaks do not vary throughout the estrous cycle (Bartlewski et al., 1999a).

### 1.3.2 Estradiol and progesterone

Luteinizing hormone binds to its receptor in theca cells of antral follicles and stimulates the production of androgens. Follicle stimulating hormone binds to its receptor in granulosa cells of the follicle to cause aromatization of these androgens to produce estradiol (Armstrong et al., 1981; Carson et al., 1979b). Each pulse of LH is followed by an increase in estradiol secretion in both cyclic and anestrous ewes (Baird, 1978; Scaramuzzi and Baird, 1977). It has been demonstrated in sheep that follicles can remain estrogenic for a maximum of 8 d when there are continuous pulses of LH of approximately 1 ng/mL every h (Dobson et al., 1997). After 8 d, even with continued treatment with LH pulses, the ability of the follicle to secrete estradiol and androstenedione is decreased (Dobson et al., 1997). Large healthy antral follicles (= 5 mm in diameter) secrete more estradiol compared to small antral follicles (Baird and Scaramuzzi, 1976; Mann et al., 1992a; McNatty et al., 1981). In cyclic ewes, as follicles grow, there is an increase in estradiol secretion reaching a maximum as the follicles reach their maximum diameter (Figure 1.1, Bartlewski et al., 1999a; Bister et al., 1999; Souza et al., 1998). During the estrous cycle, there are 3-4 peaks in serum estradiol

concentrations accompanying the three or four waves of growth of large ovarian antral follicles (see section 1.5.3) (Figure 1.1; Bartlewski et al., 1999a). However, in anestrous ewes, follicular secretion of estradiol is limited and not correlated with the growth and development of follicles (Bartlewski et al., 1998; Evans et al., 2001a; Souza et al., 1996). Similar to FSH secretion, the amplitude and duration of peaks in estradiol secretion do not vary markedly throughout the estrous cycle (Bartlewski et al., 1999a). However, during the follicular phase of the cycle, upon regression of the CL, the increase in frequency of LH pulses stimulates increased amounts of estradiol production (Baird, 1978; Hauger et al., 1977; Karsch et al., 1979; Pant, 1977; Rawlings and Cook, 1993). This enhanced production of estradiol that precedes ovulation is only marginally greater than that seen for other follicular waves in the cycle (Bartlewski et al., 1999a). There is a decrease in concentrations of estradiol and an increase in concentrations of progesterone in the follicular fluid within 16-24 h of the preovulatory LH surge (Baird, 1978; Campbell et al., 1990; England et al., 1981).

Progesterone is mainly secreted from the CL during the luteal phase of the cycle (Figure 1.2). Progesterone secretion is pulsatile and around eight pulses can be seen per day during the luteal phase of the cycle (Alecozay et al., 1988). There is no temporal association between pulses of secretion of LH and progesterone (Alecozay et al., 1988; Baird et al., 1976). The amplitude and frequency of pulses of progesterone secretion do not vary throughout the diestrous phase of the cycle (Alecozay et al., 1988). Mean daily serum progesterone concentrations rise from Day 0 (day of ovulation) to Day 11 and then reach basal levels by Day 15 after ovulation (Bartlewski et al., 1999b). It was

shown that serum progesterone concentrations are lower in prolific ewes compared to non-prolific ewes (Bartlewski et al., 1999b).

### 1.3.3 Inhibin

Inhibins belong to the transforming growth factor ß (TGF-ß) super family (Tsonis et al., 1983). Inhibins are glycoproteins consisting of an a subunit linked to either a ßA (Inhibin A) or ßB (Inhibin B) subunit by disulphide bonds (Henderson and Franchimont, 1981; Tsonis et al., 1983). Unlike estradiol, inhibins are secreted by granulosa cells of both small and large antral follicles (Campbell et al., 1991b; Mann et al., 1992a; Mann et al., 1989; Mann et al., 1992b). The mRNA expression of inhibin A and B is exclusively seen in granulosa cells and their relative expression is positively correlated to the size and estrogenicity of the follicles (Campbell and Baird, 2001; Tisdall et al., 1994). Inhibin is released in a pulsatile manner in both cyclic (Campbell et al., 1990; McNeilly and Baird, 1989; Murray et al., 1993) and anestrous ewes (Campbell et al., 1991a). It was shown that inhibin A concentrations in plasma increased as follicles grew from 2.5 mm to 4 mm in diameter during the first wave of the cycle, but did not show any temporal relationship during emergence and growth of the following waves of the cycle (Souza et al., 1998; Souza et al., 1997). In anestrous ewes, there is no temporal association of development of follicular waves and changes in serum inhibin concentrations (Evans et al, 2001a). There is an increase in inhibin secretion at the time of the preovulatory gonadotropin surge and secondary FSH surge (Campbell et al., 1990). It is believed that the removal of the negative feedback effect of estradiol on FSH secretion following ovulation is the primary cause for the secondary FSH surge. There was no association of inhibin secretion with that of FSH, estradiol or

LH secretion around this time (Campbell et al., 1990; McNeilly and Baird, 1989). However, inhibin does have a general negative feedback effect on FSH secretion (Burger and Igarashi, 1988; Henderson et al., 1988; McNeilly, 1984). Inhibin A is more potent than Inhibin B in suppressing FSH secretion from cultured sheep pituitary cells (Robertson et al., 1996).

## 1.4 Control of LH and FSH secretion

### 1.4.1 Hypothalamic regulation

Gonadotropin releasing hormone from the hypothalamus binds to its specific receptors on the gonadotrope cells of the anterior pituitary to control LH secretion (Clarke et al., 1987). Monitoring the pattern of LH secretion in the peripheral circulation and GnRH secretion in perfusates of the median eminence or portal circulation, revealed a close temporal association between GnRH and LH pulses in the ewe (Clarke and Cummins, 1982; Levine et al., 1982). The duration of the LH pulse (nadir to nadir) is about 1.5 h and peak concentrations are achieved within 10 min from the onset of an increase in LH secretion in response to GnRH (Martin, 1984). LH secretion could be used as an indirect measurement of GnRH release (Goodman, 1994). Nevertheless, there are small elevations in GnRH secretion that are not associated with LH pulses (Clarke and Cummins, 1982; Clarke and Cummins, 1987; Levine et al., 1982). It was proposed that these small pulses of GnRH secretion are required to support LH synthesis and increase secretable LH stores in the pituitary (Clarke and Cummins, 1987). The temporal association between pulsatile GnRH and LH secretion is maintained throughout the breeding (Baird, 1978; Moenter et al., 1991) and anestrous (Barrell et al., 1992; Clarke, 1988; Scaramuzzi and Baird, 1977) seasons. The amplitude

and frequency of GnRH and LH pulses are lower during anestrous season compared to the breeding season (Ciechanowska et al., 2008; Mitchell et al., 2002). In addition, in ewes, it has been demonstrated that mRNA expression levels of GnRH in the hypothalamus and GnRH receptors (GnRHr) in the hypothalamus and anterior pituitary were significantly greater during the luteal phase of the estrous cycle than in the anestrous season (Ciechanowska et al., 2008).

Pulsed GnRH secretion is important for the induction and maintenance of the release of LH and FSH from the pituitary. In sheep, it has been shown that continuous perfusion of pituitary cells with GnRH failed to stimulate the release of LH and FSH (Chakraborty et al., 1974). In a study in primates, it was shown that when GnRH pulse frequency was decreased from one an hour to one every three hours, by injections of GnRH, FSH and LH concentrations in serum were increased and decreased respectively (Wildt et al., 1981). The authors suggested that changes in GnRH pulse frequency can differentially regulate LH and FSH secretion; slower frequency of GnRH pulses favoring FSH secretion and higher frequency favoring LH secretion. However, in another study, the proportion of LH or FSH released was not influenced by either the amount or the pattern of GnRH stimulus applied to the ovine pituitary cells (McIntosh and McIntosh, 1986).

In the ewe, FSH secretion was found to be non-pulsatile during most part of the luteal phase and throughout anestrus (Wallace and McNeilly, 1986). In another study in sheep, a pulse in GnRH concentrations preceded each pulse of FSH in the pituitary venous drainage but not in the peripheral circulation (Padmanabhan et al., 1997; Van Cleeff et al., 1995). However, GnRH independent FSH pulses were noted in the portal

circulation in overiectomized ewes (Padmanabhan et al., 2003). A separate hypothalamic releasing factor for FSH has been proposed (Padmanabhan et al., 2003; Padmanabhan and McNeilly, 2001). When the effects of pulsatile GnRH secretion were blocked by treatment with a GnRH antagonist, pulsatile FSH secretion was still noted (Padmanabhan et al., 2003; Van Cleeff et al., 1995). Similarly, in overiectomized rabbits, evidence for pulsatile FSH secretion was seen in peripheral circulation after blocking the action of GnRH (Pau et al., 1991). Some researchers have suggested a hypothalamic independent regulation of FSH secretion in the ewe (Padmanabhan and McNeilly, 2001). Clarke et al.(1986) suggested that there is differential regulation of LH and FSH secretion by GnRH and that pulses of GnRH are not required to activate the release of FSH. However, McNeilly, (1995) suggested that GnRH is required to initiate FSH secretion but that only a limited exposure to GnRH is required to maintain FSH secretion. *The dependency of the FSH secretory peaks that precede follicular waves on pulsatile GnRH secretion has not been studied.*

It appears that LH and FSH are released differently from the pituitary during the estrous cycle. Only 1 to 5% of the LH stored in the pituitary is released in pulses each day, whereas FSH is only minimally stored and basal release occurs as and when it is synthesized by the pituitary (McNeilly, 1995; Taragnat et al., 1998). The secretion of FSH is constitutive; there is a tight association between the rate of synthesis and release irrespective of any kind of physiological demand (McNeilly et al., 2003). LH is stored in secretory granules and released in response to GnRH (Crawford and McNeilly, 2002; McNeilly, 1995; Taragnat et al., 1998). The mechanisms controlling these distinctive patterns of LH and FSH release are still unclear.

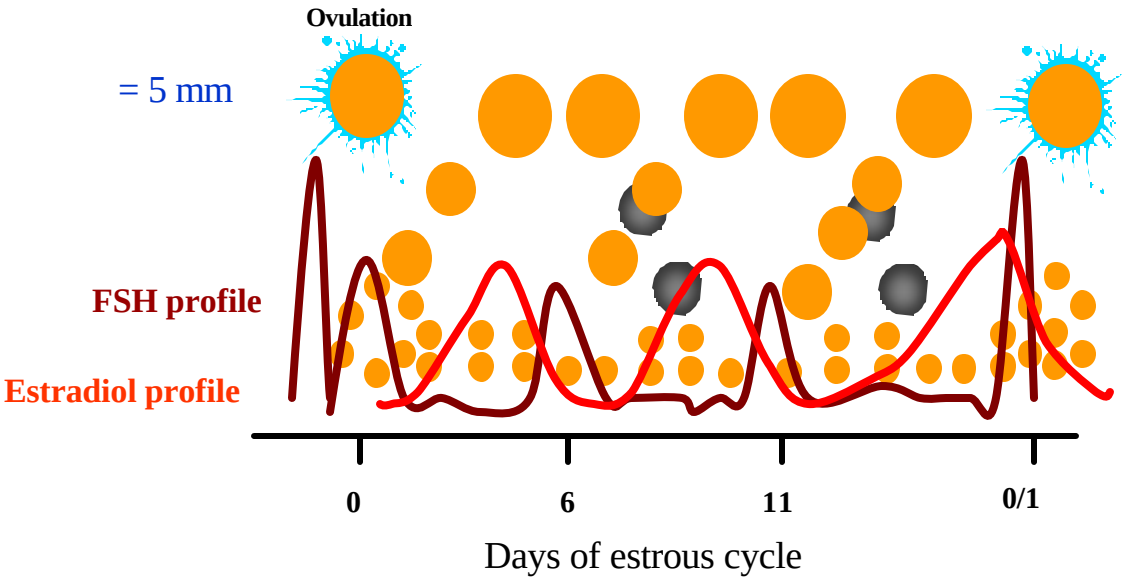


Fig 1.1. A schematic diagram showing follicular waves and FSH and estradiol profiles in serum in sheep during an estrous cycle. A follicular wave in sheep is defined as one or more follicles that emerge from a pool of small antral follicles (2-3 mm) and grow to equal to or more than 5mm in diameter before regression (black circles) or ovulation. This figure represents an estrous cycle with three waves. The emergence of each follicular wave is preceded by a peak in serum FSH concentrations (brown line). The preovulatory FSH surge is also shown. As follicles grow they secrete increasing amounts of estradiol (red line). A peak in serum estradiol concentration occurs when a follicle reaches maximum diameter (Modified from Duggavathi, 2004).

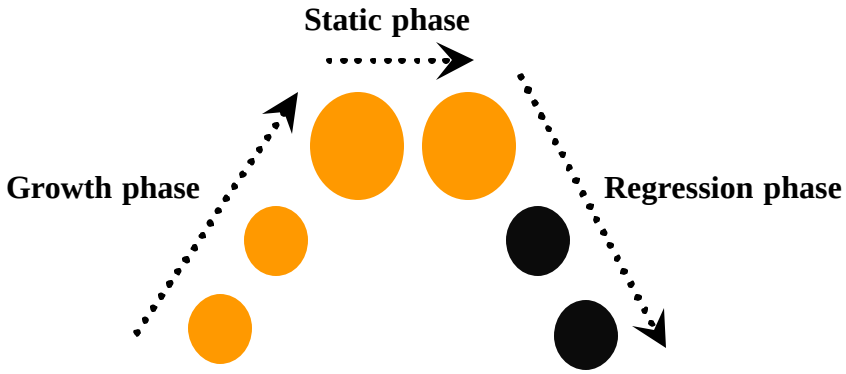


Fig. 1.1.1. Each follicular wave is comprised of 3 phases- the growth phase, the static phase, and finally the regressing phase. The growth phase of the follicle in a wave is the period between its emergence and the day that it appeared to stop its progressive increase in diameter. The static phase is from the end of the growth phase to the beginning of the regressing phase. The regressing phase is the period between the day that the follicle began its decrease in diameter to the day it reached 2 or 3 mm in diameter.

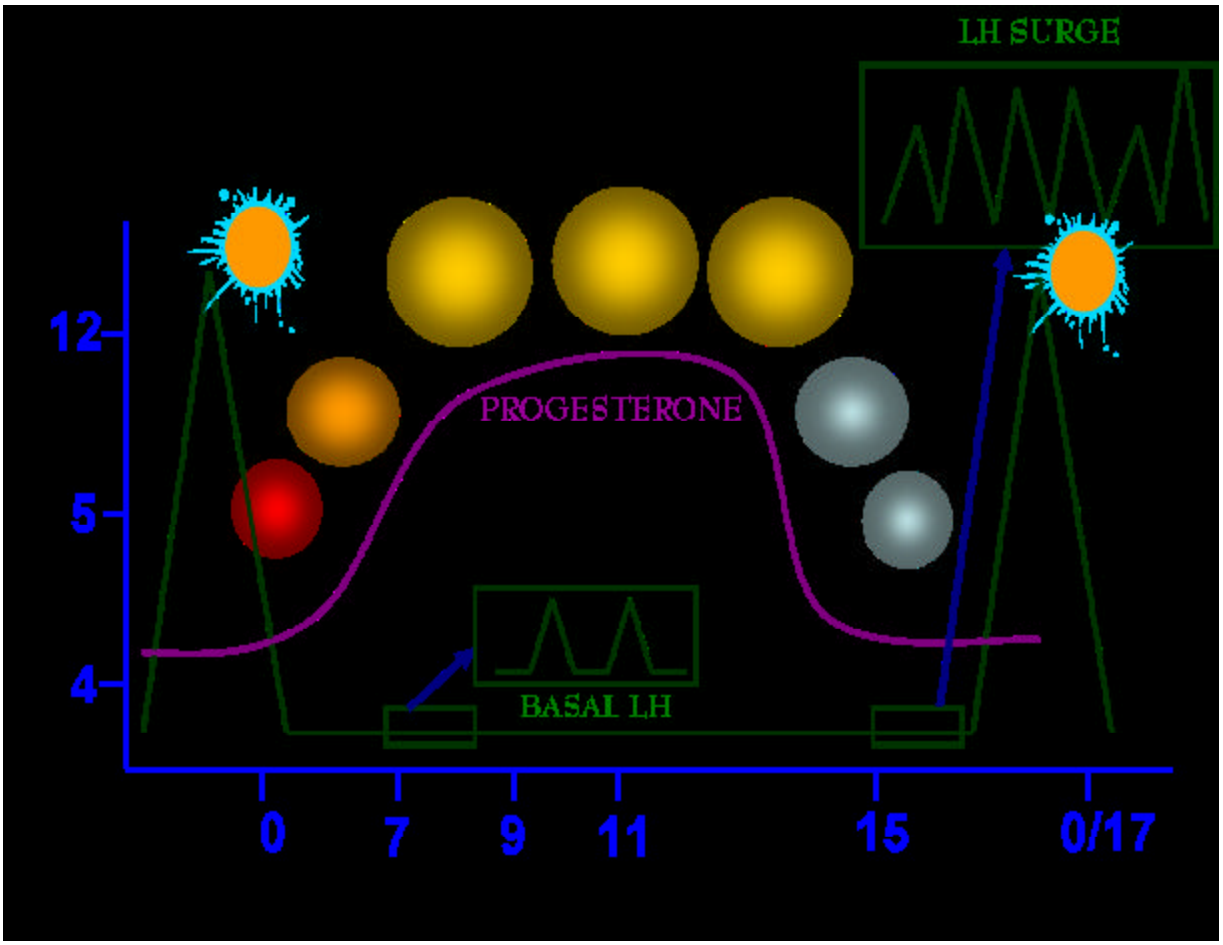


Fig. 1.2. A schematic diagram showing the temporal patterns of serum concentrations of LH and progesterone and CL diameter, during the estrous cycle in the ewe. Following ovulation, the remnant of the ovulated follicle transforms into a solid structure called the corpus luteum (colored circles). The primary purpose of the corpus luteum is to produce progesterone (purple line). The progesterone profile follows the same pattern as the diameter profile of the corpus luteum.

LH is secreted in two distinct modes (green line). There is a surge in LH secretion before each ovulation and during the rest of the cycle LH secretion is at basal levels. However, if blood samples are collected every 12 min for 6 h, pulses of LH secretion are seen (boxes).

### 1.4.2 Gonadal regulation

LH and FSH secretion is regulated by the gonadal steroids, estradiol and progesterone (Clarke, 2002). It has been established that estradiol and progesterone mediate their action through binding to their nuclear receptors (genomic action) or membrane receptors (non-genomic action) (Bishop and Stormshak, 2008; Stormshak and Bishop, 2008). The genomic action of estradiol and progesterone occur within hours to days, whereas the non-genomic occurs in seconds to min (Bishop and Stormshak, 2008). It is believed that estradiol suppresses LH pulse amplitude and progesterone LH pulse frequency (Figure 1.2; Bjersing et al., 1972; Goodman and Karsch, 1980; Karsch et al., 1979; Rawlings et al., 1984; Wheaton et al., 1984). In overiectomized ewes, treatment with luteal phase concentrations of progesterone decreased the frequency of LH pulses but not the amplitude of LH pulses (Goodman and Karsch, 1980; Rawlings et al., 1984; Tamanini et al., 1986; Thomas et al., 1988). Estradiol and progesterone operate in concert during luteal phase of the cycle to suppress the frequency of pulses of LH secretion. Estradiol exerts positive feedback effects on LH secretion during the follicular phase of the estrous cycle, when serum progesterone concentrations drop following luteal regression; in the absence of progesterone estradiol is the primary trigger for the preovulatory LH surge (Baird and Scaramuzzi, 1976; Bolt et al., 1971; Jeffcoate et al., 1984; Joseph et al., 1992; Karsch et al., 1980; Moenter et al., 1990; Rawlings et al., 1984; Scaramuzzi et al., 1970). It is believed that estradiol exerts its negative feedback effect on LH release through a non-genomic action (immediate) and a positive feedback effect (preovulatory surge in LH) through its non-genomic action (Bishop and Stormshak, 2008). The inhibitory effects of

progesterone on LH secretion are more pronounced in anestrous compared to the breeding season (Hamernik et al., 1987; Karsch et al., 1987).

In overiectomized ewes, estradiol suppressed mean serum FSH concentrations but progesterone treatment alone had no effect (Hamernik et al., 1987; Rawlings et al., 1984). Ewes treated with a combination of progesterone and estradiol had maximal suppression of mean serum FSH concentrations (Moss et al., 1981; Rawlings et al., 1984). *The regulation of basal serum FSH concentrations or the concentrations and amplitude of the peaks in FSH secretion that precede ovarian follicular waves has not been studied in the ewe.* Increased serum concentrations of estradiol and inhibin in serum, keep FSH concentrations suppressed, preceding the preovulatory FSH surge (Baird et al., 1991; Findlay et al., 1990; Pant, 1977; Wheaton et al., 1984). GnRH overrides the inhibitory effects of estradiol and inhibin on FSH secretion to induce the preovulatory FSH surge. After rupture of the largest follicle during the act of ovulation, estradiol concentrations in serum are depressed, leading to the second FSH surge (Campbell et al., 1991a; Findlay et al., 1990).

Estradiol and progesterone regulate the release of LH and FSH mainly by controlling the secretion of the GnRH at the level of hypothalamus (Arroyo Ledezma et al., 2006; Baird and Scaramuzzi, 1976; Currie et al., 1993). Secondarily, they exert their effects at the pituitary level by influencing the responsiveness of pituitary gonadotropes to GnRH (Herman and Adams, 1990). Clarke (2002) reported that estradiol could directly suppress FSH release by acting at the level of the pituitary. Estradiol enhances the inhibitory effects of progesterone on pulsatile LH secretion throughout the luteal phase of the cycle by acting mainly at the level of the hypothalamus (Goodman et al.,

1981a; Goodman and Karsch, 1980; Martin et al., 1983). In sheep, during the follicular phase of the cycle, estradiol exerts positive feedback effects on LH and FSH secretion (preovulatory gonadotropin surge) by increasing the release of GnRH from the hypothalamus and increasing the responsiveness of gonadotropes to GnRH (Herman and Adams, 1990; Moenter et al., 1990). During the follicular phase of the ovine estrous cycle, estradiol increases GnRH receptor levels (Turzillo et al., 1994) as well as estrogen receptor-alpha (ER a) levels in gonadotropes (Tobin et al., 2001). Estradiol activates the LH-secretory granules to migrate to the periphery of gonadotropes at the time of the preovulatory LH surge (Baird et al., 1981). This effect (polarization of the granules) has also been demonstrated in ovariectomized (OVX) ewes treated with single injections of estradiol benzoate (EB) (Thomas and Clarke, 1997). Estradiol fails to induce any response from the pituitary in ewes immunized against GnRH (Adams and Adams, 1986; Herman and Adams, 1990) and in ewes where the hypothalamus and hypophysis were surgically separated (Clarke and Cummins, 1984; Clarke et al., 1983; Girmus and Wise, 1992). In a recent study, in hypothalamo-pituitary disconnected ewes, it was demonstrated that ER a levels in the gonadotropes were increased by either treatment with GnRH or estradiol benzoate (EB); however, the combined treatment produced a greater effect (Clarke et al., 2005). Progesterone impedes the process by which estradiol induces the preovulatory LH surge by decreasing GnRH secretion and by decreasing the sensitivity of pituitary gonadotrophs to estradiol (Koligian and Stormshak, 1977; Legan and Karsch, 1979).

FSH secretion is also inhibited by ovarian inhibin (Baird et al., 1991; Burger and Igarashi, 1988; Henderson et al., 1988; McNeilly, 1984); this is not the case for LH. An

inverse relationship between serum inhibin A and FSH concentrations has been shown in sheep (Baird et al., 1991; Burger and Igarashi, 1988; Henderson et al., 1988; McNeilly, 1984). Immunization of ewes against inhibin resulted in greater serum concentrations of FSH than immunization against estradiol (Mann et al., 1993). Immunization of ewes against both estradiol and inhibin combined resulted in a greater increase in FSH concentrations than with either estradiol or inhibin individually (Mann et al., 1990; Mann et al., 1993). Fluctuations in daily serum concentrations of FSH and growth of follicles in follicular waves are not correlated with changes in inhibin A concentrations in cyclic and anestrous ewes (Evans et al, 2001a; Souza et al., 1998). Estradiol mainly regulates the daily fluctuations in serum FSH concentrations whereas inhibin has a more generalized suppressive effect on FSH secretion (Baird et al., 1991). *The effects of inhibin on basal serum FSH concentrations or the peaks in secretion of FSH that precede follicular waves have not been studied.*

## 1.5 Follicular development

### 1.5.1 Stages of follicular development

Follicular development is initiated during embryogenesis with regular movement of primordial germ cells to the genital ridge (Senger, 2003). Follicular development ends with either development of an antral follicle, fully competent to respond to an ovulatory stimulus from the preovulatory gonadotropin surge or undergo atresia. During prenatal or very early postnatal development, primary oocytes become surrounded by a single layer of flattened squamous cells called “pregranulosa cells” and the resultant follicle is called a primordial follicle (Figure 1.3; Greenwald and Terranova, 1988). A finite pool of primordial follicles is established during fetal

development in sheep (Juengel et al., 2002). It is believed that the primordial follicle pool (resting pool) is non-renewable and constitutes the reserve pool of follicles that an animal can use throughout its reproductive life. Lambs are born with approximately 40,000 to 300,000 primordial follicles (Driancourt et al., 1991). Nonetheless in recent studies in mice (Johnson et al., 2004) and humans (Bukovsky et al., 2004) it was shown that oogenesis and follicular renewal can occur in adult ovaries.

The recruitment of follicles from the resting pool is characterized by distinct morphological changes. Primordial follicles continuously leave the non-growing reserve pool to develop into primary or growing follicles. Primary follicles consist of an oocyte surrounded by cuboidal somatic cells called "granulosa cells" (Figure 1.3). The granulosa cells begin to proliferate from this stage, and express markers of cell proliferation, such as proliferating cell nuclear antigen (PCNA; (Fortune, 2003; Wandji et al., 1996). Primary follicles mature to become secondary follicles as multiple layers of granulosa cells surround the oocyte (Figure 1.3; Driancourt, 2001; Fortune, 2003). At this stage, the follicle begins to acquire theca cells from the mesenchymal cells of the stroma of the ovary; theca cells form the outer layer of the follicle. In addition, the oocyte grows larger and secretes a glycoprotein rich substance called the Zona Pellucida (Figure 1.3; ZP), this surrounds the oocyte and separate the oocyte from the granulosa cells. Also at this stage, there is formation of gap junctions between granulosa cells and development of a plexus of capillaries within the theca layer. The majority of the primordial follicles undergo atresia by apoptosis. The numbers of primordial follicles decrease by about 20% in the fetal lamb from the middle of pregnancy to parturition

(Driancourt et al., 1991). After birth, around 35% of follicles are lost due to atresia during the life span of the ewe (Driancourt et al., 1991).

The secondary follicles develop into early antral or tertiary follicles (Figure 1.3; Lundy et al., 1999). The formation of an antrum leads to two distinct subsets of granulosa cells having different morphological and functional characteristics; cumulus granulosa cells surrounding the oocyte and the mural granulosa cells located at the periphery of the follicle (Cran et al., 1979). A mature follicle is called a Graafian or preovulatory follicle (Figure 1.3; Hay and Moor, 1975). Antral follicle development is a fast process, which takes only a few days compared to weeks and months for primary and secondary follicle growth. In the ewe, the time taken for the primordial follicle to develop to the mature graafian follicle stage is about 16 to 26 weeks (Cahill and Mauleon, 1980; Campbell et al., 2003). The time taken by the primordial follicle to develop to the early preantral follicle stage (0.2 mm in diameter) is approximately 130 d (Cahill and Mauleon, 1980; Cahill et al., 1981), while the time needed for the follicle to grow from 0.2 mm to 0.5 mm is only 24 to 35 d (Turnbull et al., 1977). Development of follicles from 0.5 mm to 2.2 mm in diameter requires only 5 d and the final growth of follicles to a preovulatory follicle diameter (4.5 to 5 mm in diameter) takes about 4 d (McNeilly, 1984; Turnbull et al., 1977).

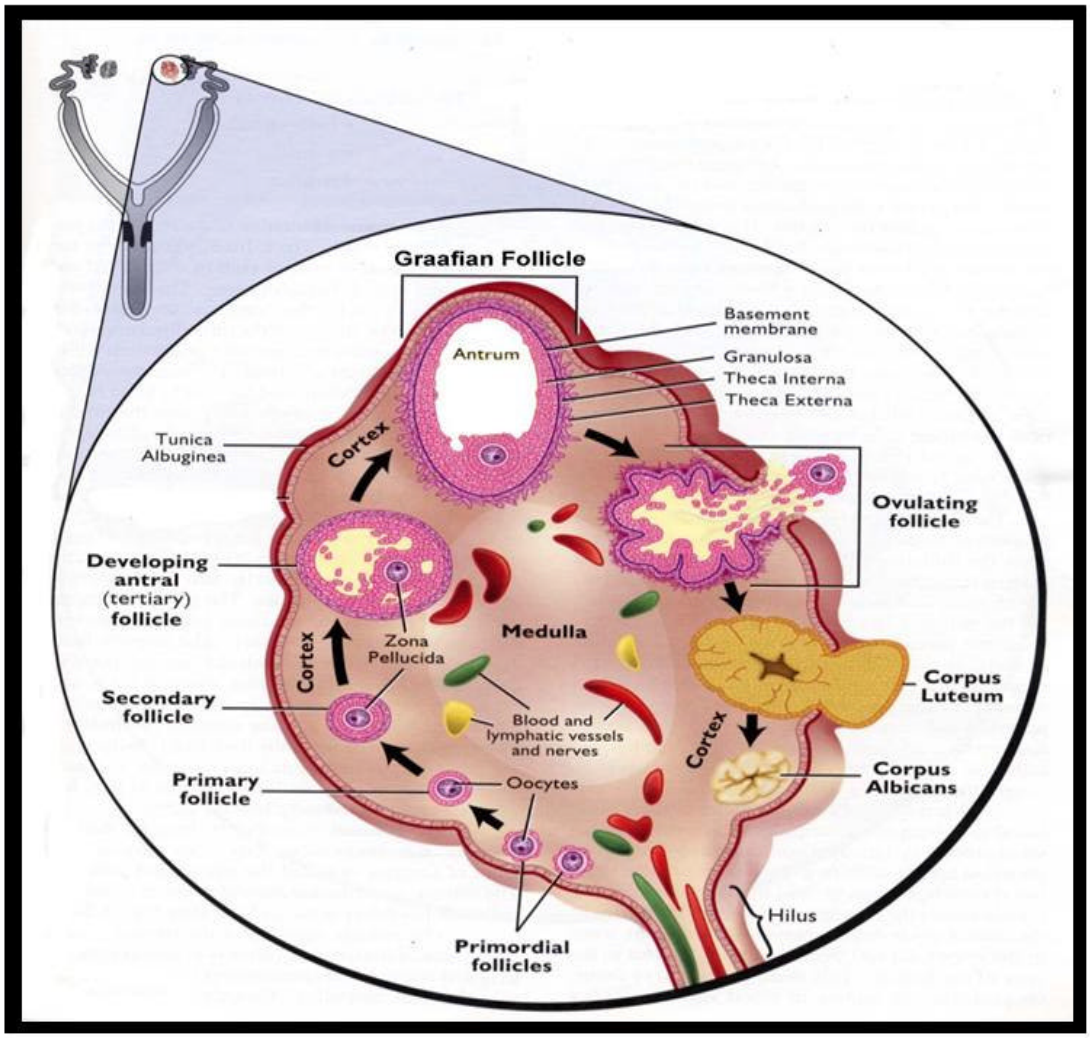


Fig. 1.3. A schematic diagram showing major structures of the ovary. The cortex houses all stages of follicles such as primordial follicles, primary follicles, secondary follicles, tertiary follicles or graafian follicles. It also houses the corpus luteum or the corpus albicans. Adapted from Senger, 2003. Used by permission.

### 1.5.2 The early stages of follicular development

The initial period of follicular development comprises the growth of follicles from primordial to early antral stage. According to McNatty et al. (1999), the initial period of follicular development can be dived into 5 phases:- types 1 (primordial), 1a (transitory), 2 (primary), 3 and 4 (preantral) and 5 (early antral) based on the morphological characteristic of granulosa cells and the number of layers of granulosa cells.

The early stages of follicular development are generally considered to be gonadotropin independent (McNatty et al., 1981). However, the regulation of early follicular development is not very well understood. In mice, O'Shaughnessy et al. (1997), primordial follicles do not express LH and FSH receptors while in cattle (Xu et al., 1995) and sheep (Tisdall et al., 1995), it has been shown that FSH receptors (FSHr) appear on granulosa cells of primary follicles. Fortune et al. (1999) demonstrated that even though primary follicles express FSHr; these receptors are not coupled to adenyl cyclase. The role of LH in the development of early stage follicles is not clear (Fortune, 2003). It has been suggested that responsiveness to LH and FSH is acquired by the follicles at the end of this early stages of follicular development i.e when the follicle has reached 2-3 mm in diameter (Campbell et al., 1995). Attainment of gonadotropic dependence is very important for subsequent antral follicular development.

### 1.5.3 Development of antral follicles: Follicular wave model

With the use of transrectal ultrasonography, it has been shown that ovarian antral follicles emerge and grow in a regular pattern in ruminants (Adams, 1999; Adams and Jaiswal, 2008; Adams et al., 2008). This pattern of follicular development is called

the wave pattern of follicular growth (Figure 1.1; Adams et al., 2008; Ginther and Kot, 1994; Schrick et al., 1993; Sirois and Fortune, 1988). Each follicular wave is comprised of 3 phases; the growing phase, the static phase, and finally the regressing phase (Figure 1.1.1; Adams, 1999; Bartlewski et al., 1999a). The growing phase of the follicle in a wave is the period between its emergence and the day that it appeared to stop its progressive increase in diameter. The static phase is from the end of the growth phase to the beginning of the regressing phase. The regressing phase is the period between the day that the follicle began its decrease in diameter to the day it first reached the same diameter seen at emergence (Bartlewski et al., 1999a; Ravindra et al., 1994). Follicular wave emergence refers to the commencement of synchronous onward growth and development of a group of small antral follicles that eventually gain the ability to ovulate or undergo atresia (Ginther et al., 2003). Recruitment of follicles refers to that group of antral follicles that initiate growth in a wave (Ginther et al., 2003). In most species, only a few of those follicles recruited escape atresia and continue to develop to an ovulatory diameter and this process is known as selection (Ginther et al., 2003). Selected follicles (dominant follicles) will grow further in a favorable endocrine milieu, suppressing other follicles (subordinate follicles) in a wave. This feature is referred to as dominance (Ginther et al., 2003).

In cattle, the first observation of two or three waves of follicular development in an estrous cycle was reported by (Rajakoski, 1960). Two to three decades later, these observations were clarified with the help of improved techniques such as transrectal ultrasonography and cattle were described as having two or three follicular waves per cycle (Adams, 1999; Ginther et al., 1989; Knopf et al., 1989; Savio et al., 1988; Sirois

and Fortune, 1988). The emergence of a follicular wave in cattle is distinguished by a significant increase in the number of follicles (6-9 follicles) in the 4-6 mm size range; (Ginther et al., 1996) and is preceded by a transient peak in plasma FSH concentrations (Adams et al., 1992). Recently, it was shown in cattle that even smaller follicles (1-3 mm in diameter) emerge and grow in a wave like pattern (Jaiswal et al., 2004). The growth rate of follicles in a wave is similar over several days after emergence but eventually one follicle develops to be a dominant follicle (deviation) and this dominant follicle suppresses the growth of other follicles (subordinate follicles) (Ginther et al., 1989). There is also a reduction in the number of small follicles in the ovary and thus suppression of subsequent follicular wave emergence, during the development of the dominant follicle (Ginther et al., 1996). Deviation occurs approximately 3 d following the emergence of the wave when the dominant follicle reaches 8 mm in diameter (Ginther et al., 1996). The dominant follicle grows at a faster rate compared to subordinate follicles and the duration of this event (deviation) is = 8 h (Ginther et al., 1996). The phenomenon of follicular dominance was well established in cattle through a series of studies involving cauterization of the dominant follicle and treatment with protein fractions from follicular fluid (Adams et al., 2008; Adams et al., 1992). Cauterization of a dominant follicle led to a peak in FSH secretion and emergence of a follicular wave (Adams et al., 1992; Ko et al., 1991). Administration of follicular fluid suppressed FSH concentrations and delayed emergence of a follicular wave (Adams et al., 1992; Quirk and Fortune, 1986). Cessation of follicular fluid treatment caused an FSH peak and a follicular wave to occur within 24 h (Adams et al., 1992).

In cattle, the ovulatory follicle always develops from the final wave of the estrous cycle (Ginther et al., 1996). The characteristics of cycles with two or three follicular waves have been thoroughly studied in cattle. Estrous cycles with two waves were shorter (20.4 +/- 0.3 d) compared to those having three waves (22.8 +/- 0.6 d) (Ginther et al., 1989). The dominant follicle of the ovulatory wave differed significantly between cows with two or three waves per cycle. The day of emergence (Day 9.6 +/- 0.2 and 16.0 +/- 1.1), the length of the interval from emergence of the follicle to ovulation (10.9 +/- 0.4 and 6.8 +/- 0.6 d), and diameter on the day before ovulation (16.5 +/- 0.4 and 13.9 +/- 0.4 mm) also differed significantly between cows with two or three waves per cycle (Ginther et al., 1989). In a recent study, it was confirmed that in cattle, cycles with two waves were shorter compared to cycles with three waves (19.8 ± 0.2 *versus* 22.5 ± 0.3; Jaiswal et al., 2009). The authors from the latter study also reported that the there was two-fold greater chance of repeatability of a particular wave pattern in the estrous cycle within a herd in any season of the year than cycles that changed patterns (70% *versus* 30%; Jaiswal et al., 2009). It was also concluded that the duration of follicular dominance (the growth and static phases of the dominant follicle) of wave 1 of the cycle regulates the number of waves during an estrous cycle in cattle. Follicular dominance was 3 d longer in cycles with two compared to three waves (Jaiswal et al., 2009)

In sheep, before the use of transrectal ultrasonography, the development of ovarian antral follicles was studied using post-mortem examinations, laproscopy or endoscopy (Brand and de Jong, 1973; Hutchinson and Robertson, 1966; McNatty et al., 1984a; McNatty et al., 1984b; Noel et al., 1993). Based on a study that used India or

carbon ink to track the development of large follicles, it was suggested that three or more cohorts of antral follicles developed during the estrous cycle (Smeaton and Robertson, 1971). In another study, sheep were slaughtered and ovaries were collected at different time points throughout the estrous cycle; macroscopic examination of the ovaries revealed the presence of two follicular waves (Brand and de Jong, 1973). Further, laparoscopic examination of 8 Suffolk ewes for 18 d, in different seasons of the year, demonstrated three waves of follicle growth in both the anestrous and breeding seasons (Noel et al., 1993). In recent years, the advent of ultrasonography led to great advancement in our understanding of follicular waves in cyclic, anestrous and pregnant ewes (Bartlewski et al., 1998; Ginther and Kot, 1994; Ginther et al., 1995; Ravindra et al., 1994; Schrick et al., 1993; Souza et al., 1998).

A follicular wave is defined differently in sheep compared to cattle. A follicular wave in sheep is defined as one to four follicles that emerge or grow from a pool of small follicles (2 or 3 mm in diameter) to reach an ovulatory follicle diameter of = 5 mm before ovulation or regression (Figure 1.1), with emergence limited to a 24 h period (Duggavathi et al., 2003). Two to four waves per cycle have been reported in different breeds of sheep (Bartlewski et al., 1999a; Contreras-Solis et al., 2008; Evans et al., 2000; Ginther et al., 1995; Noel et al., 1993; Ravindra et al., 1994). *The characteristics of waves in cycles with different number of waves have not been studied in sheep. The mechanisms regulating the number of follicular waves during the estrous cycle is not clear.* Follicular waves emerge more frequently during the estrous cycle in sheep than in cattle; every 4 to 5 d in sheep (Bartlewski et al., 1998; Evans et al., 2000; Ginther et al., 1995) *versus* 7 to 10 d in cattle (Knopf et al., 1989; Savio et al., 1988; Sirois and

Fortune, 1988). These waves are preceded by a peak in serum FSH concentrations in both cyclic and anestrous ewes (Figure 1.1; Bartlewski et al., 1999a; Bartlewski et al., 1998; Bister et al., 1999; Duggavathi et al., 2004; Duggavathi et al., 2003; Ginther et al., 1995; Souza et al., 1998). Unlike cattle, in sheep, all follicles recruited in a wave can grow and reach an ovulatory size, suggesting the absence of selection and deviation in sheep (Bartlewski et al., 1999a; Duggavathi et al., 2004). The largest follicle growing in the first wave of the cycle may have a longer lifespan compared to other waves during an estrous cycle (Bartlewski et al., 1999a). Depending on the breed of sheep, the largest follicle growing in a wave can attain a maximum diameter of 4 to 7 mm; the maximum follicle diameter is lower in prolific ewes compared to non-prolific ewes (Bartlewski et al., 1999a; Evans et al., 2002; Hunter et al., 2004; Souza et al., 1998). The ovulatory follicle usually emerges from the final wave of the cycle; however, in prolific breeds such as Finn and Rambouillet X Booroola ewes, 50% of the ovulatory follicles emerge from the penultimate wave of the cycle (Bartlewski et al., 1999a; Gibbons et al., 1999). Serum progesterone concentrations in prolific Finn ewes are much lower compared to non-prolific Western White Face ewes (Bartlewski et al., 1999b). Treatment of non-prolific Western White Face ewes with PGF2a and medroxy progesterone acetate (MAP) at midcycle altered follicular dynamics and increased ovulation rate by approximately 50% by causing ovulations from both the penultimate and final wave of the cycle (Bartlewski et al., 2003). In a subsequent field study (Davies, 2005), MAP and PGF2 alpha treatment in non prolific Western White Face ewes did not result in an increase in lambing rate. *The effect of the ovulation of aged follicles on fertility in sheep is not clear (Evans, 2003a). Therefore, it would be*

*worthwhile to examine the differences in the quality of follicles with different lifespans before ovulation.*

## 1.6 Gonadotropic regulation of follicular development

Gonadotropins play a very important role in control of emergence and growth of antral follicles (Baird and McNeilly, 1981; Ireland, 1987; Picton et al., 1990b). It is believed that antral follicles beyond 2-3 mm in diameter are dependent on gonadotropins (Campbell et al., 1998; McNeilly et al., 1982; Picton et al., 1990a). In sheep, it appears that FSH receptors are found in the granulosa cells as early as the primary follicle stage and LH receptors are found in the theca cells of preantral follicles (Logan et al., 2002; Tisdall et al., 1995). The numbers of FSH and LH receptors begin to increase as antral follicles grow to 2 mm in diameter (Carson et al., 1979a). Initially, LH receptors are present only in theca cells of follicles. As antral follicles reach 4 mm in diameter, LH receptors are also seen on granulosa cells (Logan et al., 2002). It appears that estradiol and FSH promote the production of LH receptors on granulosa cells of large antral follicles in ewes (England et al., 1981). During the final stages of follicular development and prior to the preovulatory LH surge, FSH concentrations in serum are very low and LH concentrations in serum are high (Baird and McNeilly, 1981). Therefore, it was suggested that the early and final stages of development of large follicles are FSH and LH dependent respectively (Campbell et al., 1995).

In ewes given a long term treatment with a GnRH agonist, it was shown that FSH alone, but not LH alone, could promote follicles to grow from 2-3 mm in diameter to a preovulatory size (= 5 mm; Campbell et al., 1999; Picton et al., 1990b). It has been demonstrated that in ewes treated with follicular fluid or inhibin or a GnRH antagonist,

there was a sudden suppression and atresia of ovulatory follicles (Baird et al., 1990; Campbell et al., 1999; Campbell et al., 1991a; Henderson et al., 1986; McNeilly, 1984). In FSH and FSHr knock-out mice and in conditions of FSH mutations occurring naturally in humans, there was no growth of follicles beyond the preanatral stage (Huhtaniemi, 2000; Kumar, 2005). Recently, it was suggested that FSH regulates the rate of growth of preantral follicles (Campbell et al., 2004). Further, in GnRH antagonist-treated ewes, when treatment with FSH was withdrawn in the presence of continued treatment with LH, growth of preovulatory follicles was sustained in 50-55% of ewes (Campbell et al., 1999). In LH and or LH receptor (LHr) knock-out mice, there was normal preantral follicle development; but, healthy antral preovulatory follicles were absent and abnormal antral follicles containing degenerative oocytes were seen (Ma et al., 2004; Zhang et al., 2001). In GnRH agonist treated ewes, FSH stimulated follicle development was inhibited by treatment with frequent injections of high-amplitude low frequency pulses of LH (Picton et al., 1990b). These observations suggest that both FSH and LH are important in the control of antral follicle development to a preovulatory size. It should be noted that the majority of studies above depended on single point observations prior to the application of ultrasonography and also did not consider the peaks in serum concentrations of FSH that precede follicular waves.

The numbers of small follicles (2-3 mm in diameter) remain constant throughout the estrous cycle in the ewe except during the periovulatory period (Figure 1.1; Duggavathi et al., 2003). The large growing follicles of each wave in sheep do not appear to suppress the growth of small follicles as seen in cattle (dominance)

(Driancourt et al., 1991; Duggavathi et al., 2004; Duggavathi et al., 2005a). It has been demonstrated in sheep that the superovulatory response was not affected by the presence or absence of a large healthy follicle (Driancourt et al., 1991). Also, when small follicles were co-cultured with large follicles, there was no change in cell division (incorporation of H-3 thymidine in granulosa cells) of small follicles compared to when small follicles were cultured alone (Driancourt et al., 1991). These observations raise the question as to whether follicular dominance exists in sheep. Nevertheless, there are reports suggesting the presence of follicular dominance in sheep (Gonzalez-Bulnes et al., 2001; Gonzalez-Bulnes et al., 2004; Gonzalez-Bulnes and Veiga-Lopez, 2008). The presence of a large follicle in one ovary in the ewe was found to decrease the recruitment and growth of other follicles in the either ovary (Gonzalez-Bulnes et al., 2004; Gonzalez-Bulnes and Veiga-Lopez, 2008). It was also shown that there was a greater decrease in the number of small follicles growing to larger sizes in the ovary containing a dominant follicle compared to the contralateral ovary (Gonzalez-Bulnes et al., 2004). As in cattle, transient peaks in serum FSH concentrations precede each emergence of a follicular wave in both cyclic and anestrous ewes (Figure 1.1; Bartlewski et al., 1999a; Bartlewski et al., 1998; Bister et al., 1999; Duggavathi et al., 2004; Duggavathi et al., 2003; Ginther et al., 1995; Souza et al., 1998). Previous studies in sheep have shown that small follicles (2-3 mm in diameter) are capable of responding to FSH peaks, created by the administration of ovine FSH (oFSH), to give rise to a new follicular wave (Barrett et al., 2006; Duggavathi et al., 2005a). Doubling the amplitude of endogenously driven FSH peaks by injecting oFSH, did not affect the number of follicles in a wave or the growth of follicles (Duggavathi et al., 2005a). FSH treatment

was capable of initiating waves if given at any phase of the previous follicular wave in the ewe (Duggavathi et al., 2005a; Duggavathi et al., 2004). It was also observed that follicles growing in such induced follicular waves had a normal growth phase and lifespan and produced normal serum concentrations of estradiol (Duggavathi et al., 2005a; Duggavathi et al., 2004). In addition, the rhythm of endogenously driven waves was not altered by the induction of a new wave by injection of oFSH (Duggavathi et al., 2005a; Duggavathi et al., 2004). Again, the existence of follicular dominance in the ewe is very questionable.

Subcutaneous estradiol ($E_2$) and or progesterone ($P_4$) releasing implants have been used extensively (Barrett et al., 2006; Barrett et al., 2007; Barrett, 2007) to study the regulation of gonadotropin secretory patterns and antral follicular dynamics in anestrous and cyclic ewes. In anestrous ewes, treatment with $E_2$ releasing implants abolished LH pulsatality and prevented follicle wave emergence without affecting FSH secretion (Barrett, 2007). Based on this study, it was concluded that although peaks in FSH secretion initiate follicular waves in the anestrous ewe, wave emergence also requires some level of pulsed LH secretion. *It would be interesting to see whether restoration of LH pulses would re-establish follicular waves in the treatment model above.* In cyclic ewes, treatment with $E_2$ releasing implants resulted in truncation of FSH peaks and prevented follicle wave emergence. However, injection of physiological concentrations of oFSH, in ewes given implants for 10 d, reinitiated follicle wave emergence (Barrett et al., 2006). In the latter study, pulsed LH secretion was not affected and the estradiol-17ß implants had little impact on mean and basal serum FSH concentrations. It was also noted that maintenance of the 2-3 mm follicle pool did not

appear to require regular secretion of FSH peaks. However, in another study in cyclic ewes, $E_2$ treatment for 20 d resulted in truncated FSH peaks, a lack of wave emergence, and a reduced small follicle pool (Barrett et al., 2007). Injection of physiological concentrations of oFSH did not reinitiate follicular wave emergence in the latter study (Barrett et al., 2007). *The role of FSH peak concentrations and amplitude and basal FSH secretion (peaks and pulses excluded) in regulating ovine antral follicle wave dynamics and maintenance of the small follicle pool is still not clear in sheep and needs further investigation. The whole issue of follicular dominance in the ewe requires further clarification.*

In a previous study (Duggavathi et al., 2005b), it was shown that none of the parameters of pulsatile LH secretion varied significantly with either the emergence of the final follicular wave of a cycle or with the end of the growth phase of the largest follicle of the penultimate wave of the cycle in ewes. However, a significant increase in LH pulse frequency and mean and basal LH concentrations was seen after the end of the growth phase of the largest follicle of the final follicular wave, coinciding with functional luteolysis. Therefore, it was concluded that LH secretory patterns were associated with luteolysis, but not with the growth and demise of the antral follicles in a wave. This is interesting, as there are changes in LH pulse frequency around the time of follicular deviation (establishment of the dominant follicle) in follicular waves in cattle (Ginther et al., 1998). In cattle, suppression of LH pulse frequency decreases growth of the dominant follicle post-deviation, but deviation does not require this increase in pulsed LH secretion (Ginther et al., 2001). It has been shown that although the temporal patterns of progesterone concentrations during an estrous cycle alter the LH secretory

patterns, follicular waves at all stages of the cycle in the ewe are similar in duration and maximal follicle size (Bartlewski et al., 1999a; Duggavathi et al., 2003). In another study (Bartlewski et al., 2000a), an increase in LH pulse amplitude occurred with the onset of the static phase of the first wave of the cycle. However, this was probably related to the progesterone driven decrease in pulse frequency at the onset of the luteal phase. Recently, in ewes treated with ovarian transplants and given a GnRH antagonist to suppress pulsed LH secretion, it was demonstrated that growth of antral follicles was similar in ewes subsequently given no further treatment, pulses of LH or constant infusion of LH (Campbell et al., 2007). In that study, it was concluded that the pulsatile mode of LH secretion is not required for ovulatory follicle development (Campbell et al., 2007). Nevertheless, there are reports that suggest the dependence of ovarian follicles on pulsed LH secretion. For example, creation of sub-luteal serum concentrations of progesterone in the ewe was associated with increased LH pulse frequency and the lifespan of the largest follicle of the first wave of the ovine estrous cycle was prolonged (Vinoles et al., 1999). *The role of pulsatile LH secretion in emergence and growth of follicular waves is still unclear and needs further investigation.*

It has been suggested that CL exerts both systemic and local effects on follicular dynamics throughout the estrous cycle (Bartlewski et al., 2001; Contreras-Solis et al., 2008). Recently, in ewes, it was shown that follicles growing in wave 2 of the cycle (i.e during the mid luteal phase of the cycle) had diminished growth phase, lifespan, growth rate and maximum follicle diameter compared to follicles growing in wave 1 (early luteal phase) and wave 3 (late luteal phase) of the cycle (Contreras-Solis et al., 2008). It

has also been demonstrated that the effect of dominance (suppression of the number of small subordinate follicles and wave emergence) by dominant follicles was greater in wave 1 and the ovulatory wave compared to the follicles growing during the mid-luteal phase of the cycle in ewes (Contreras-Solis et al., 2008). This absence of dominance during the mid-luteal phase was believed to be due to local suppressive effects of progesterone from the CL (Adams, 1999; Gonzalez-Bulnes et al., 2005). Local effects of the CL have been suggested previously (Bartlewski et al., 2001; Contreras-Solis et al., 2008). In humans, oocytes obtained from the follicles growing in the ovary contralateral to the ovary where ovulation occurred are healthier and produce good quality embryos at the time of invitro fertilization (IVF) (Fukuda et al., 1996).

There is an increase in pulsatile LH secretion when rams are introduced to cyclic ewes at any stage of the estrous cycle (Hawken et al., 2007). In contrast, in cycling goats, the buck effect on pulsed LH secretion was only seen when bucks were introduced during early and late luteal phase of the cycle, but not during the mid-luteal phase (Hawken et al., 2009). The authors suggested that high serum progesterone concentrations during the mid-luteal phase blocked the male effect in goats (Hawken et al., 2009). In another study, it was reported that there was an almost two-fold increase in mean LH concentrations after exposure of anestrous ewes to a group of rams and estrous ewes (Ferreria et al., 2008).

## 1.7 Angiogenesis of ovarian follicles

Angiogenesis is the process of formation of new blood vessel from already existing vasculature which involves enzymatic degradation of the basal membrane of pre-existing vessels, and migration and proliferation of endothelial cells (Redmer et al.,

2001a; Redmer and Reynolds, 1996; Stouffer et al., 2001). Angiogenesis occurs throughout follicular development to provide a supply of regulatory hormones and the nutrients required for the growth and development of follicles, oocytes, as well as the corpus luteum that forms after ovulation of the follicle (Fraser and Lunn, 2000; Hunter et al., 2004; Redmer and Reynolds, 1996). Angiogenesis is considered to be a limiting step in the maturation and selection of the preovulatory follicle (Stouffer et al., 2001). It is believed that preantral follicles do not have their own blood supply and depend on blood vessels in the surrounding stroma for survival (Bruno et al., 2009b). Recently, Martelli et al. (2009) reported that in the pig preantral follicles acquire their own vascular supply when they reach 110 µm in diameter. In ovarian antral follicles of primates, the theca layer acquires capillary networks during the formation of the antrum and this production of blood vessels is regulated by angiogenic factors (Stouffer et al., 2001).

Several pro-angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2) and endothelial nitric oxide synthase (NOS3), have been identified in ovarian follicles of several species (Bruno et al., 2009b; Grazul-Bilska et al., 2006; Reynolds et al., 2002). Pro-angiogenic factors in ovarian follicles are involved in promoting migration and proliferation of endothelial cells, and or acting as survival factors for endothelial cells, increasing vascular permeability, promoting antrum formation of the follicle, and events leading to ovulation (Redmer and Reynolds, 1996; Tamanini and De, 2004). Using doppler ultrasonography in cattle, it was shown that the largest dominant follicle has greater vascularity compared to second largest subordinate follicle, suggesting that maintenance of an adequate blood

supply to the follicle is required for the establishment of follicular dominance (Acosta, 2007). In addition, in the preovulatory follicle, around the time of ovulation, LH surge increases the blood flow within the follicle wall (Acosta, 2007). Inhibition of angiogenesis during the estrous cycle leads to profound suppression of ovarian function (Bruno et al., 2009b; Fraser and Duncan, 2009). Reduced thecal vascularity is one of the first signs of follicular atresia (Bruno et al., 2009b; Jablonka-Shariff et al., 1996). It was recently shown that VEGF maintains follicular ultrastructural integrity and advances follicular growth in goats (Bruno et al., 2009a). In non-human primates, endothelial cell proliferation were blocked after inhibition of VEGF during recruitment or selection of follicles; therefore, affecting follicular development (Fraser and Duncan, 2009). In addition, inhibition of VEGF synthesis during the early luteal phase limits the development of luteal microvasculature (Redmer et al., 2001b; Redmer and Reynolds, 1996). Expression of VEGF and NOS3 in granulosa and theca layers were greater in dominant, estrogen-active follicles than nondominant, estrogen–inactive follicles of the first follicular wave in cattle. Endothelial nitric oxide synthase is known to affect follicular development, steroidogenesis and maturation of the oocyte through its potent angiogenic and vasodialatory effects (Grazul-Bilska et al., 2006; Reynolds et al., 2002). In various species, FGF-2 has been shown to promote growth of primordial and primary follicles, granulosa and theca cell proliferation as well as oocyte survival (Nilsson et al., 2001; Wandji et al., 1996; Zhou and Zhang, 2005). Antiapoptotic effects of FGF-2 have been demonstrated in porcine granulosa cells (Grasselli et al., 2002).

## 1.8 Gap junctions in the ovary

Gap junctions are cell-to-cell channels, which directly connect the cytoplasmic compartment of neighbouring cells (Figure 1.4, 1.5; Gershon et al., 2008; Grazul-Bilska et al., 1997b; Kidder and Mhawi, 2002). They have a hexameric structure known as connexon, which are assemblies of proteins termed connexins (Cx; Figure 1.4; Gershon et al., 2008; Grazul-Bilska et al., 1997b; Kidder and Mhawi, 2002). Gap junctions facilitate exchange of nutrients, ions, and regulatory molecules of less than 1kDa, such as calcium ions, cAMP, inositol 1, 4, 5-triphosphate, between two contacting cells (Gershon et al., 2008; Grazul-Bilska et al., 1997b). Connexins are named according to their sizes. Multiple connexins including Cx26, Cx32, Cx37, Cx43 and Cx45, are expressed within the ovarian follicle, in some cases within the same cell type (Gershon et al., 2008; Grazul-Bilska et al., 1997b). Intercellular communication through gap junctions is required for normal ovarian folliculogenesis (Grazul-Bilska et al., 1997b; Kidder and Mhawi, 2002). Connexin 37 is the major Cx that builds gap junctions between the oocyte and the ovarian granulosa cells, whereas Cx43 is the main gap junction protein forming the channels within the granulosa cells and theca layers (Figure 1.5; Kidder and Mhawi, 2002; Simon and Goodenough, 1998). It is known that gap junctions are involved in the transfer of cyclic adenosine monophosphate (cAMP) from the granulosa cells to the oocyte; this maintains the oocyte in meiotic arrest. After the preovulatory LH surge, there is breakdown of gap junctional communication within the ovarian follicle interrupting the supply of cAMP from the granulosa cells to the oocyte. This lead to reinitiation of meiosis (Dekel, 1988; Edry et al., 2006).

The expression of Cx43 is influenced by the stage of follicular and luteal development (Grazul-Bilska et al., 1998; Grazul-Bilska et al., 1997b). It was observed that Cx43 is present only in the granulosa layer in preantral follicles whereas in antral follicles, Cx43 was present in both granulosa and theca cells (Grazul-Bilska et al., 1997b). In sheep, larger antral follicles express more Cx43 than small and medium sized antral follicles (Grazul-Bilska et al., 1997b). In atretic follicles of rats and sheep, expression of Cx26, Cx32, or Cx43 was decreased or completely absent in granulosa cells, but expression of Cx43 was still present in theca cells (Wiesen and Midgley, Jr., 1994). In cattle, it was observed that unlike Cx43 expression, which was present in granulosa and theca cells of some atretic follicles, Cx32 expression was seen in every atretic follicle (Grazul-Bilska et al., 1997b). It was suggested that Cx32 expression could be a marker of atretic follicles in cattle (Grazul-Bilska et al., 1997b). In ewes, when the expression for Cx43 was examined in luteal tissue, it appeared to be greatest during the early and mid luteal phase but decreased during the late luteal phase of the estrous cycle (Grazul-Bilska et al., 1996). However, in another study, it was noted that Cx43 expression remained at high levels during the time of luteal regression in sheep (Borowczyk et al., 2006), indicating that gap junctional communication was necessary for the transfer of the luteolytic signal between the luteal cells (Grazul-Bilska et al., 1997a; Grazul-Bilska et al., 1997b). Further, a positive correlation of progesterone secretion with that of changes in Cx43 mRNA expression was demonstrated in cultured ovine luteal cells (Borowczyk et al., 2007). These observations suggest that gap junctions are involved in the control of steroidogenesis in the ovine corpus luteum. Through knockout mouse models, connexins have been shown to have pro-cell survival

roles and to maintain granulosa cell proliferation (Ackert et al., 2001; Kidder and Mhawi, 2002). In mice ovaries lacking Cx43, it has been demonstrated that there is a decrease in follicular growth and an increase in granulosa cell apoptosis (Ackert et al., 2001; Gittens et al., 2005). Determination of the expression of Cx43 in ovarian follicles and CL could be a good predictor of development status and quality.

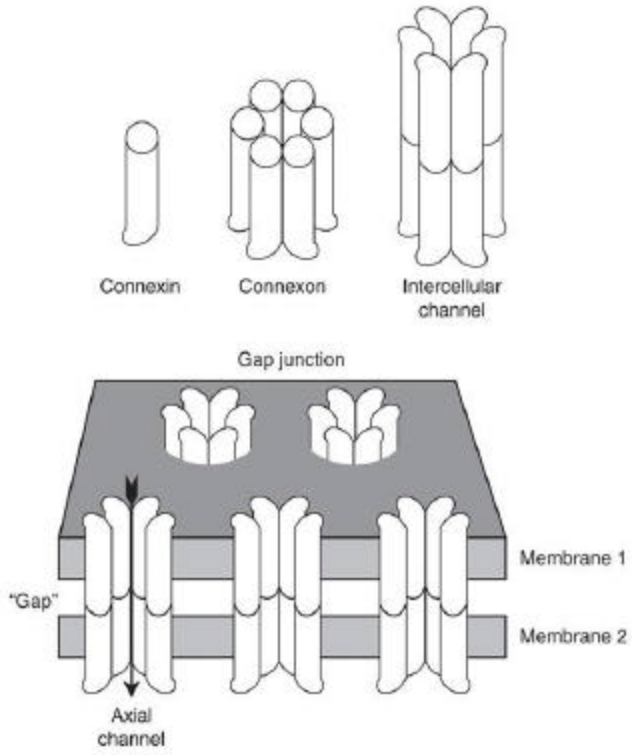




Fig. 1.4. A diagram showing the structure of the gap junction. Gap junctions have a hexameric structure known as connexon, which are assemblies of proteins called connexins. Connexons from one cell dock with connexons in an adjacent cell, to form cell-to-cell channel, which directly connects the cytoplasmic compartment of neighboring cells. Adapted from Goodenough and Paul, 2009. Used by permission

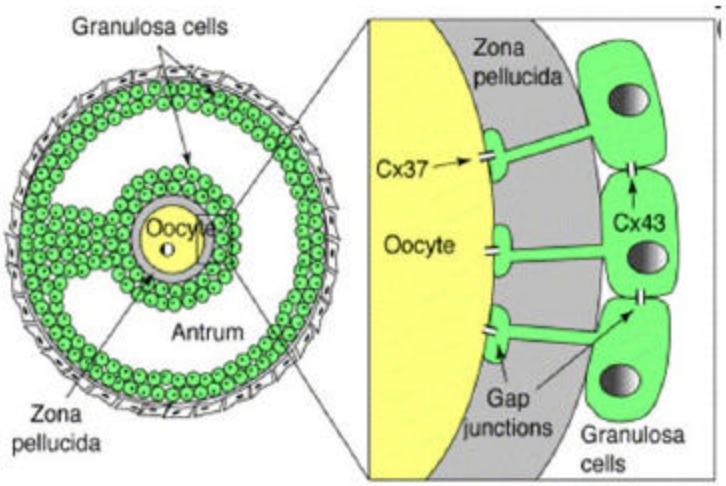


Fig. 1.5. Gap-junction-mediated coupling between cells of the ovarian follicle. Granulosa cells are coupled to each other by Cx43-containing gap junctions. In addition, the cumulus granulosa cells extend processes through the zona pellucida and make gap junctions with the oocyte. These oocyte–granulosa gap junctions contain Cx37. Adapted from Simon and Goodenough, 1998. Used by permission

## 1.9 General objectives

Studies described in this thesis were performed to explore the gonadotropic and steroidal regulation of follicular development in cyclic and anestrous ewes. Studies were further conducted to investigate the mechanism of regulation of the number of follicular waves during an estrous cycle and to assess the quality of aged follicles by examining certain markers of follicular development. The following are the general objectives described in this dissertation:-

1. We are not aware of any previous study comparing hormone profiles and characteristics of large antral follicles in follicular waves for ewes with differing numbers of waves per cycle. Such observations could help to reveal important regulatory relationships. Therefore, as our first objective, follicular waves, and serum FSH, estradiol and progesterone profiles were characterized and compared amongst cyclic Western White Face ewes with three or four follicular waves per cycle. An additional objective was to examine the temporal association between the growth characteristics of follicular waves and the serum FSH, estradiol and progesterone concentrations during the estrous cycle.

2. In anestrous ewes, large estradiol-releasing implants completely abolished LH pulsatility and blocked follicular wave emergence (Chapter 3; Barrett, 2007). Interestingly, in these ewes, FSH secretion was only minimally affected. It was concluded that although peaks in FSH secretion initiate follicular waves in the anestrous ewe, this also requires some level of pulsed LH secretion. The purpose

of the present experiment was to replace LH pulses by using frequent GnRH injections in the experimental model above in anestrous ewes, to see if restored LH pulsatility would in fact allow restoration of follicular waves. This would confirm that although peaks in FSH secretion initiate follicular waves in the anestrous ewe, some level of pulsed LH secretion is also required. Therefore our second objective was to examine the need for pulsed LH secretion for the emergence and growth of ovarian follicular waves in anestrous ewes.

3. To try and clearly define the role of pulsatile LH secretion in the regulation of follicular waves in cyclic ewes, two experiments were designed to alter LH pulse frequency over a wide range without affecting the FSH peaks that herald follicular waves. Our third objective was to increase LH pulse frequency, during the midluteal phase of a cycle, to that seen in the follicular phase and to suppress LH pulse frequency to a value less than that seen in the normal luteal phase and see the effects on emergence and growth of follicular waves.

4. Treatment of anestrous ewes with estradiol-releasing implants completely abolished LH pulsatility, blocking follicular wave emergence (Chapter 3; Barrett, 2007). Interestingly, in these ewes, FSH secretion was only minimally affected and the pool of small ovarian follicles was not changed. Based on this study, it was concluded that although peaks in FSH secretion initiate follicular waves in the anestrous ewe, this also requires some level of pulsed LH secretion. Data presented in Chapter 2 of the present thesis, also showed that when pulsed

secretion of LH was restored by GnRH injections in anestrous ewes, given the estradiol-releasing implants, follicular wave emergence resumed. Our fourth objective was to treat anestrous ewes with implants releasing progesterone and estradiol to see if the combined treatment would reduce the amplitude of FSH peaks and in turn reduce the numbers of small follicles in the ovary as was seen with a similar treatment in the breeding season (Barrett et al., 2007).

5. FSH secretion in the ewe appears less acutely dependent on GnRH secretion than the secretion of LH is. In the ewe the mechanisms that regulates the FSH peaks that precede follicular waves is not known. Therefore, our fifth objective was to determine how neutralization of GnRH affects the peaks of FSH secretion that precede follicular waves and the intervening basal serum concentration of FSH in the anestrous ewe.

6. Treatment of ewes with PGF2a and MAP during mid cycle, increased ovulation rate by causing ovulations from both the penultimate and final wave of the cycle. Preovulatory follicles in the penultimate wave are older compared to follicles in the final wave of the cycle. Fertility of aged follicles is not clear. However, a field study which utilized the above treatments to enhance ovulation rate determined that there was no improvement in fertility. Therefore, our sixth objective was to evaluate the expression of (a) markers of angiogenesis; VEGF and NOS3 (b) a marker of endothelial cells and thus vascularization; Factor VIII (c) a marker of gap junctional communication: Connexin 43 (Cx43) and (d) a

marker of cellular proliferation: PCNA in preovulatory follicles obtained from the penultimate and final waves of the estrous cycle in Western White Face (WWF) ewes treated with PGF2a and MAP as described above.

## 1.10 Hypotheses

1. Ewes with three or four waves of follicular development per cycle would differ with respect to follicular growth characteristics and endocrine profiles.
2. Although the frequency of pulses of LH secretion is very low in anestrous ewes, they are still essential for the emergence and growth of ovarian follicular waves.
3. Increasing or decreasing LH pulse frequency over the normal range seen during the luteal phase would not influence any aspect of ovarian follicular waves.
4. Estradiol and progesterone in combination would suppress the secretory peaks of FSH secretion and reduce the numbers of small antral follicles in the ovary in the anestrous ewe.
5. Immunization against GnRH would block the pulsatile secretion of LH and follicular waves but allow continued peaks in FSH secretion in anestrous ewes.
6. The lack of increased fertility in non-prolific WWF ewes, where ovulation was increased by PGF2a and MAP sponge treatment, could be partly explained by decreased angiogenesis and limited cellular proliferation and gap junctional interactions in preovulatory follicles from the penultimate wave compared to the final wave of an estrous cycle.

# Chapter 2: OVARIAN ANTRAL FOLLICULAR DYNAMICS IN SHEEP REVISITED: COMPARISON AMONGST ESTROUS CYCLES WITH THREE OR FOUR FOLLICULAR WAVES [*]

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## 2.1 ABSTRACT

In this study, the characteristics of follicular waves and the pattern of FSH, estradiol and progesterone concentrations in serum were compared between cycles with three (n=9) or four (n=10) follicular waves in Western white face (WWF) ewes. Transrectal ultrasonography and blood sampling were performed daily during one cycle. Estrous cycles were 17.11±0.3 and 17.20±0.2 d long in cycles with three and four waves, respectively (P>0.05). The first inter-wave interval and the interval from the emergence of the final wave to the day of ovulation were longer in cycles with three compared to four waves (P<0.05). The growth phase (5.1±0.5 vs. 3.1±0.4 d) and lifespan (5.67±0.3 vs. 4.3±0.3 d) of the largest follicle growing in the last or ovulatory wave was longer in cycles with three compared to four waves (P<0.05). The maximum diameter of the largest follicle was greater in the first wave and the ovulatory wave compared to other waves of the cycle (P<0.05). The regression phase of the largest follicle growing in the first wave was longer in cycles with three compared to four waves (4.44±0.4 vs. 3.4±0.4 d; P<0.05). The length of the lifespan, regression phase, and although not significant in every case, FSH peak concentration and amplitude, decreased across the cycle (P<0.05). We concluded that estrous cycles with three or four follicular waves were confined within the same length of cycle in WWF ewes. In this study, there were no apparent endocrine or follicular characteristics that could

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explain the regulation of the different number of follicular waves (three or four waves) during the cycle of similar length.

## 2.2 INTRODUCTION

In the ewe, ovarian antral follicles emerge or grow, from a pool of small antral follicles (2-3 mm in diameter), in a wave like pattern, every four to five days, reaching =5 mm in diameter before regression or ovulation (Bartlewski et al., 1999a; Evans et al., 2000; Ginther et al., 1995; Schrick et al., 1993; Souza et al., 1996; Souza et al., 1998). There are normally three or four such follicular waves per estrous cycle and each wave is preceded by a peak in serum concentrations of FSH (Bartlewski et al., 1999a; Duggavathi et al., 2003; Evans et al., 2000; Ginther et al., 1995; Souza et al., 1996; Souza et al., 1998). The mechanisms regulating the number of follicular waves in each cycle in sheep are still unclear. There are several studies in cattle in which researchers have reported the characteristics of estrous cycles with two or three waves (Celik et al., 2005; Ginther et al., 1989; Noseir, 2003). In this sense, Ginther et al. (1989) concluded that estrous cycles with two waves were shorter (20.4 ± 0.3 d) compared to those associated with cattle having three waves per cycle (22.8 ± 0.6 d). The dominant follicle of the last/ovulatory wave differed significantly between cows with two or three waves per cycle not only in the day of emergence (9.6 ± 0.2 and 16.0 ± 1.1 d, respectively), but also in the length of the interval from emergence of the follicle to ovulation (10.9 ± 0.4 and 6.8 ± 0.6 d, respectively), and diameter on the day before ovulation (16.5 ± 0.4 and 13.9 ± 0.4 mm, respectively; Ginther et al., 1989). In another recent study in cattle (Jaiswal et al., 2009), it was confirmed in cattle, that cycles with two waves were shorter compared to cycles with three waves (19.8 ± 0.2 *versus* 22.5 ± 0.3 d,

respectively). Based on that study, the authors concluded that the duration of follicular dominance of wave 1 regulated the number of waves during the estrous cycle (Jaiswal et al., 2009).

Souza et al. (1998) concluded that in the first ovarian follicular wave of the estrous cycle in sheep, changes in follicular size were accompanied by an increase in the serum concentrations of ovarian steroids and inhibin A. However, there were no changes in serum concentrations of steroids and inhibin A during the second and third follicular waves; the changes in follicle diameter were similar to the first wave. Based on the results of the previous study (Souza et al., 1998), the authors suggested that fluctuations in both inhibin A and estradiol secretion controlled the FSH peak preceding the first follicular wave of the cycle, the cause of the FSH fluctuations associated with waves two and three of the cycle remain unclear. Bartlewski et al. (1999a) observed that the amplitude and duration of peaks in serum concentrations of estradiol-17ß and FSH did not change over the entire estrous cycle.

We are not aware of any previous study comparing hormone profiles and characteristics of large antral follicles in follicular waves for ewes with differing numbers of waves per cycle. Such observations could help to reveal important regulatory relationships. Therefore, in the present study, follicular waves, and serum FSH, estradiol and progesterone profiles were characterized and compared between estrous cycles with three (n = 9) or four (n = 10) follicular waves in cyclic WWF ewes. Further, an additional objective of the present study was to examine the temporal association between the growth characteristics of follicular waves and the serum FSH, estradiol and progesterone concentrations during the estrous cycle. We hypothesized

that cycles with three or four waves of follicular development would differ with respect to follicular growth characteristics and endocrine profiles.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Animals

Care and handling of experimental animals were done according to the Canadian Council on Animal Care's published guidelines. Sexually mature, clinically healthy, cycling, WWF ewes (5-7 years of age) were kept outdoors in sheltered paddocks. Ewes were fed with a maintenance diet of hay; cobalt iodized saltlicks and water were freely available. The WWF is a cross between the Columbia and Rambouillet breeds. Ewes were monitored daily for estrus with vasectomized crayon marker-harnessed rams.

### 2.3.2 Ultrasound technique

Ovarian antral follicular dynamics were monitored in all ewes by transrectal ovarian ultrasonography (scanning) using a 7.5-MHz linear transducer stiffened with a hollow plastic rod and connected to a B-mode, real-time echo camera (Aloka SSD-900, Overseas Monitor, Richmond, BC, Canada). This technique has been validated for monitoring ovarian antral follicular dynamics and for CL detection in sheep [4;7;12]. All images were viewed at a magnification of X 1.5 with constant gain and focal point settings. Ovarian images were recorded using a Video Cassette Recorder (Panasonic AG 1978 VCR, Matsushita Electric, Mississauga, ON, Canada) on high-grade video tapes (Fuji S-VHS, ST-120 N, Fujifilm, Tokyo, Japan) for later examination. The relative position and dimension of follicles and luteal structures were also recorded using ovarian maps.

### 2.3.3 Blood sampling

Blood samples (10 mL) were collected by jugular venipuncture into Vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA). All samples were allowed to clot at room temperature for 18 to 24 h. Samples were then centrifuged for 10 min at 1500 x g, and serum was removed and stored at !20 $^{o}$C until assayed.

### 2.3.4 Experimental design

Nineteen ewes underwent daily transrectal ultrasonography of ovaries starting from the day of estrus, and continuing for one estrous cycle, to monitor ovarian antral follicular development. Ulrasonography began at 8AM each day. Blood samples were taken daily, prior to ultrasonography, throughout the experimental period, for hormone estimations. Each day, during scanning, the number, diameter and relative position of all the follicles = 1 mm in diameter and corpora lutea were mapped. From the data obtained in the follicular maps, the growth profile of each antral follicle in a wave was determined. Based on the pattern of growth profiles, we categorized ewes into two groups. One group which had three waves per cycle (n=9) and the second group which had four waves per cycle (n=10).

### 2.3.5 Analysis of data for ovarian follicles and corpora lutea

A follicular wave consisted of a follicle or a group of follicles that emerged and grew from 2 or 3 mm in diameter to = 5 mm (growth phase), before regressing to 2 or 3 mm in diameter (regression phase) or ovulation; time spent at =5mm was regarded as the static phase (Bartlewski et al., 1999a; Bartlewski et al., 1998). The length of the growth, static and regression phases, and life span of the largest follicle growing in each follicular wave were analyzed for the estrous cycle studied. The lifespan of a large

antral follicle was defined as the interval from its emergence at 2 or 3 mm to its regression back to 2 or 3 mm. If more than 1 follicle attained the same maximum size, the follicle that reached the maximum diameter first and/or remained at its maximum size for the longest period of time, was regarded as the largest follicle of the wave. The interval between the emergences of two waves was termed the inter-wave interval (IWI). In this study, estrous cycles with three waves (three wave cycles) had two inter-wave intervals and cycles with four waves (four wave cycles) had three inter-wave intervals. The interval from emergence of the first follicular wave to the second follicular wave was abbreviated IWI-1/3 and IWI-1/4 for three and four wave cycles, respectively; the second to the third wave IWI-2/3 and IWI-2/4 for three and four wave cycles, respectively; and the third to the fourth wave in four wave cycles IWI-3/4. In addition, the interval from emergence of the last or ovulatory wave of the cycle, to the day of ovulation at the end of the cycle, was compared to the other inter-wave intervals. We believed this was an acceptable IWI to study as emergence of the first wave of the next cycle is expected on or within one day of the day of ovulation (Bartlewski et al., 1999a). This final interval of the cycle was abbreviated IWI-3/3 for three wave cycles and IWI-4/4 for four wave cycles. Ultrasonography was not continued long enough to characterize wave 1 of the estrous cycle following the cycle studied, and hence the exact day of wave emergence could not be determined. The number of small (=1 to =3 mm in diameter), medium (4 mm in diameter), and large follicles (= 5 mm in diameter) and maximum follicle diameter each day, in each of the inter-wave intervals were analyzed for the estrous cycle studied. Ovulation was detected with ultrasonography as the disappearance of a large follicle that had been followed in its growth/static phase for

several days. Data for ovarian follicles and corpora lutea were integrated for both ovaries of each ewe.

### 2.3.6 Hormone analysis

Progesterone (Rawlings et al., 1984), estradiol (Joseph et al., 1992) and FSH (Currie and Rawlings, 1989) concentrations were measured in serum samples taken daily using radioimmunoassay procedures. The assay sensitivities, defined as the lowest concentration of a hormone capable of significantly displacing radio-labeled hormone from the antibody were: 0.03 ng/mL for progesterone, 1.0 pg/mL for estradiol and 0.1 ng/mL for FSH. The ranges of standards were: 0.1 to 5 ng/mL, 1.0 to 100 pg/mL, and 0.12 to 16.0 ng/mL for the progesterone, estradiol and FSH assays, respectively. A concentration equivalent to the sensitivity of the assay was assigned to serum samples with hormone concentrations lower than the assay sensitivity.

The intra- and inter-assay coefficients of variation (CVs) were 11.8% and 14.5% or 8.0% and 12.5% for reference sera with mean progesterone concentrations of 0.31 or 1.28 ng/mL, respectively. The intra- and inter-assay CVs were 9.6% and 13.1% or 7.2% and 9.4% for reference sera with mean estradiol concentrations of 7.70 or 23.10 pg/mL, respectively. The intra- and inter-assay CVs were 2.5% and 2.7% or 3.7% and 3.8% for reference sera with mean FSH concentrations of 1.27 or 3.46 ng/mL, respectively.

Peaks in FSH concentrations in blood samples taken daily were identified using cycle-detection software (Clifton and Steiner, 1983). A fluctuation or cycle was defined as a progressive rise and fall in hormone concentrations that encapsulated a peak concentration, i.e. nadir-to-peak-to-nadir (Clifton and Steiner, 1983). Mean basal FSH concentrations were determined by averaging the lowest points between peaks (nadirs).

Follicle stimulating hormone peak concentration was defined as the concentration of FSH observed at the apex of the FSH peak. Follicle stimulating hormone peak amplitude was defined as the difference between the FSH peak concentration and the nadir before the peak concentration. Follicle stimulating hormone peak duration was defined as the interval between the two nadirs encompassing the FSH peak.

### 2.3.7 Statistical analyses

The differences between groups (cycles with three or four waves) were analyzed for each wave of the cycle using generalized estimating equations and modeling each outcome separately as a continuous measure in a model with a fixed effect for group, wave, and a first-order interaction term (group by wave) (PROC GENMOD, SAS for Windows ver 9.1, SAS Institute Inc., Cary, NC). Correlations between observations resulting from repeated measures within animals were accounted for by a repeated statement in the model for ewe identification modeled using an autoregressive (AR(1)) correlation structure. The same model was also used to examine differences in each outcome between waves within each group. The linear regression model was used to examine the associations between the difference in progesterone concentrations from Day 0 to 4 with FSH peak concentrations and day of FSH peak. The t-test was used to compare length of estrous cycles and ovulation rate amongst groups of ewes with three or four waves per cycle. Statistical significance was defined as $P<0.05$. Data are expressed as mean ± S.E.M.

## 2.4 RESULTS

### 2.4.1 Antral follicle development and ovulations

The mean lengths of estrous cycles were 17.11 ± 0.3 and 17.20 ± 0.2 d for three-wave and four-wave cycles, respectively (P>0.05). Follicular waves emerged on Day 0.2 ± 0.4, 6.0 ± 0.3 and 10.4 ± 0.2 in three-wave cycles (Waves 1, 2 and 3, respectively) and on Day –0.3 ± 0.2, 4.4 ± 0.2, 8.1 ± 0.3 and 11.9 ± 0.3 in four-wave cycles (Waves 1, 2, 3 and 4, respectively; Day 0 = the day of ovulation; Figure 2.1). Except for Wave 1, the days of emergence of follicular waves differed between three and four wave cycles (P<0.05; Figure 2.1).

The first inter-wave interval (IWI-1/3 and IWI-1/4) and the interval from the emergence of the final wave to the day of ovulation (IWI-3/3 and IWI-4/4 ) were longer in three-wave compared to four-wave cycles and were also longer than intervening inter-wave intervals for both groups of ewes (P<0.05; Table 2.1).

The growth phase of the largest follicle growing in the last or ovulatory wave of the cycle was longer in three-wave compared to four-wave cycles (P<0.05; Figure 2.2A). The longest static phase was seen for the largest follicle growing in Wave 2 (P<0.05; Figure 2.2B). The lengths of the follicular lifespan and of the regression phase decreased from the first to the last waves of the cycle (P<0.05; Figure 2.2C and 2.2D) and the lifespan of the largest follicle growing in the final or ovulatory wave of the cycle was longer in three-wave compared to four-wave cycles (P<0.05; Figure 2.2D). The regression phase of Wave 1 was significantly longer in three-wave compared to four-wave cycles (P<0.05; Figure 2.2C).

The number of small follicles did not differ between inter-wave intervals and or amongst groups (P>0.05; Table 2.1). The number of medium sized follicles was greatest for IWI-2/3, IWI-3/3 and IWI-3/4 compared to other inter-wave intervals (P<0.05; Table 2.1). The number of large follicles was low in IWI-1/4, IWI-1/3 and IWI-3/3 compared to other inter-wave intervals (P<0.05; Table 2.1). The mean daily maximum diameter of the largest follicle recorded in a wave was greatest for IWI-1/3, IWI-1/4, IWI-3/3 and IWI-4/4 compared to intervening inter-wave interval in both groups of ewes (P<0.05; Table 2.1). The ovulation rates were 1.78 ± 0.2 and 2.1 ± 0.3 d for three-wave and four-wave ewes, respectively (P > 0.05).

### 2.4.2 Characteristics of serum FSH concentrations

Peaks in serum FSH concentrations occurred on Day 0.0 ± 0.2, 5.78 ± 0.1 and 10.44 ± 0.2 in three-wave cycles (Waves 1, 2 and 3, respectively) and on Day –0.1 ± 0.1, 4.3 ± 0.2, 8.1 ± 0.2 and 12.4 ± 0.3 in four-wave cycles (Waves 1, 2, 3 and 4, respectively; Day 0 = the day of ovulation; Figure 2.1). Except for the first FSH peak, the days of occurrence of FSH peaks differed (P < 0.05) between three-wave and four-wave cycles.

Based on blood samples collected daily, the FSH-peak concentration, amplitude and duration did not differ between the three-wave and four-wave cycles when peaks were compared between cycles for the equivalent wave (P>0.05; Figure 2.3A, 2.3B and 2.3D). However, basal serum FSH concentrations for the FSH peak triggering the ovulatory wave were higher in three-wave compared to four-wave cycles (P<0.05; Figure 2.3C). Although not significant in every case, both FSH peak concentration and amplitude declined across the estrous cycle (Figure 2.3). Basal serum FSH

concentrations for the FSH peak triggering the ovulatory wave were lower compared to basal FSH concentrations for all other peaks for four-wave cycles ($P<0.05$; Figure 2.3C). FSH peak duration increased from the first to the second FSH peak within three-wave cycles ($P<0.05$; Figure 2.3D). Within four-wave cycles, FSH peak duration was greater for the second FSH peak compared to first and third FSH peak; FSH peak duration was also greater for the FSH peak triggering the ovulatory wave compared to the first FSH peak ($P<0.05$; Figure 2.3D).

### 2.4.3 Serum estradiol concentrations recorded on daily basis

During the estrous cycle, mean serum estradiol concentrations did not differ between inter-wave intervals or amongst groups ($P>0.05$; Table 2.1). Estradiol peak concentration and amplitude did not differ between the groups; however, in three- and four-wave cycles, although not significant in every case, estradiol peak concentration and amplitude was greater for the ovulatory wave compared to other waves of the cycle (Figure 2.4).

### 2.4.4 Serum progesterone concentrations and corpora lutea recorded on a daily basis

When plotted for days of the estrous cycle, mean serum progesterone concentrations did not differ between cycles with three or four waves of follicular development ($P>0.05$). When plotted for the days of each IWI and normalized to wave emergence, mean serum progesterone concentrations in IWI-1 were found to be greater at the end of the IWI-1 in three-wave compared to four-wave cycles ($P<0.05$; Figure 2.5, Table 2.1). However, mean serum progesterone concentration for the first four days of the IWI-1 did not differ between three-wave and four-wave cycles ($P>0.05$; Figure

2.5). The increase or difference in serum progesterone concentrations from Day 0 to 4 of the IWI-1 did not differ between cycles with three and four waves ($P>0.05$; Figure 2.5). Linear regression analysis did not reveal any association between the increase or difference in serum progesterone concentrations from Day 0 to Day 4 of the IWI-1 with the peak in serum FSH concentration preceding the second wave of the cycle or day on which this peak occurred ($P>0.05$; Figure 2.5).

The mean number of CL's ($1.78 \pm 0.2$ vs $2.00 \pm 0.2$) and mean diameter of CL's ($9.58 \pm 0.6$ vs $9.25 \pm 0.5$ mm) throughout the estrous cycle did not differ amongst cycles with three or four follicular waves ($P>0.05$).

Table 2.1: Inter-wave intervals (IWI), the mean numbers of follicles in various size categories recorded daily, mean daily maximum follicle diameter and mean daily estradiol and progesterone concentrations in estrous cycles with three (n=9) or four (n=10) follicular waves in cyclic Western White Face ewes.

| End points | Three-wave cycles | | | Four-wave cycles | | | |
|---|---|---|---|---|---|---|---|
| | IWI-1/3 | IWI-2/3 | IWI-3/3 | IWI-1/4 | IWI-2/4 | IWI-3/4 | IWI-4/4 |
| Inter-wave interval | 5.78 ± 0.5 $^{ax}$ | 4.44 ± 0.4 $^{y}$ | 6.56 ± 0.2 $^{ax}$ | 4.70 ± 0.2 $^{bx}$ | 3.70 ± 0.3 $^{y}$ | 3.80 ± 0.2 $^{y}$ | 5.10 ± 0.3 $^{bx}$ |
| Mean daily number of small follicles (= 1 mm to = 3 mm in | 12.43 ± 0.8 | 14.47 ± 1.5 | 13.12 ± 1.5 | 13.99 ± 0.6 | 12.97 ± 0.6 | 14.44 ± 1.4 | 14.0 ± 1.7 |
| Mean daily number of medium follicles (4 mm in diameter) | 0.89 ± 0.1 $^{x}$ | 1.33 ± 0.2 $^{y}$ | 1.33 ± 0.2 $^{y}$ | 0.74 ± 0.2 $^{x}$ | 1.04 ± 0.2 $^{x}$ | 1.7 ± 0.3 $^{y}$ | 1.07 ± 0.1 $^{x}$ |
| Mean daily number of large follicles (= 5 mm in diameter) | 1.44 ± 0.2 $^{x}$ | 2.33 ± 0.3 $^{y}$ | 1.67 ± 0.2 $^{x}$ | 1.2 ± 0.1 $^{x}$ | 1.9 ± 0.2 $^{y}$ | 2.2 ± 0.2 $^{y}$ | 1.7 ± 0.2 $^{y}$ |
| Mean daily maximum follicle diameter (mm) | 6.78 ± 0.3 $^{x}$ | 5.4 ± 0.2 $^{y}$ | 6.78 ± 0.3 $^{x}$ | 6.2 ± 0.4 $^{x}$ | 5.3 ± 0.2 $^{y}$ | 5.5 ± 0.2 $^{y}$ | 6.2 ± 0.3 $^{x}$ |
| Mean daily estradiol concentration (pg/mL) | 3.48 ± 0.3 | 3.06 ± 0.3 | 3.56 ± 0.3 | 4.06 ± 0.3 | 3.7 ± 0.5 | 3.63 ± 0.4 | 4.06 ± 0.4 |
| Mean daily progesterone concentrations (ng/mL) | 0.82 ± 0.2 $^{ax}$ | 2.31 ± 0.2 $^{y}$ | 1.25 ± 0.1 $^{z}$ | 0.43 ± 0.1 $^{bx}$ | 1.84 ± 0.3 $^{y}$ | 2.18 ± 0.3 $^{y}$ | 0.92 ± 0.2 $^{z}$ |

Data are presented as mean ± S.E.M.

$^{a, b}$ P<0.05; Different superscript letters within a row denote significant differences between the groups within each inter-wave interval.
$^{x, y, z}$ P<0.05; Different superscript letters within a row denote significant differences between the periods of scanning within the groups.

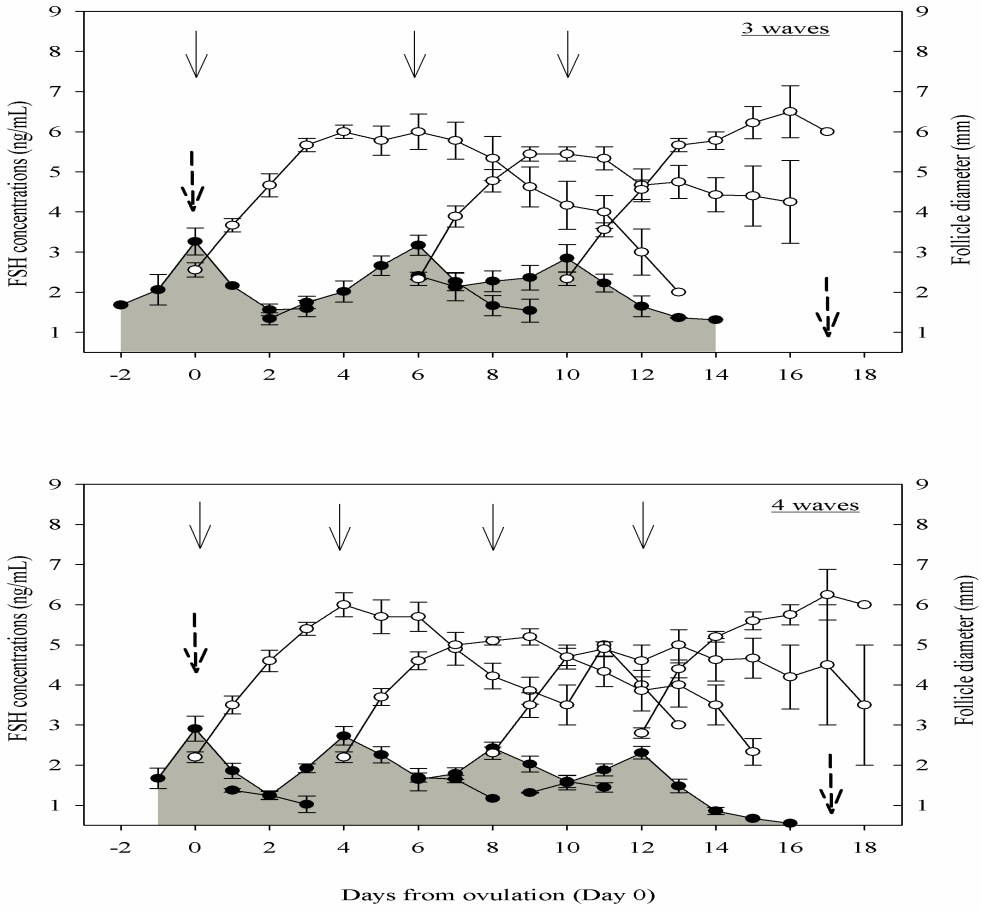


Fig 2.1. Peaks in serum concentrations of FSH (outlined with shading) and their associated emerging follicular waves (open circles) in three- (top panel; n=9) or four- (bottom panel; n=10) wave cycles. Data were normalized to the day of ovulation at the start of the experiment (Day 0). Concentrations of FSH and follicle diameters are expressed as mean ± S.E.M. The average curves representing the growth, static and regression phases of all ewes in a group were normalized for each follicle wave to the mean day of wave emergence (indicated by the solid arrows; (Barrett et al., 2006)). All FSH peaks for all ewes are shown normalized to the mean day of occurrence of the apex of the FSH peak for each wave. For every FSH peak, serum concentration profiles were delimited by the encompassing nadirs of the FSH concentrations (hence the overlap of the data for adjacent peaks in some cases). Day of ovulation at the start and end of the estrous cycle is indicated by broken arrows.

It is important to note that the characteristics of antral follicles shown in Table 1 and Figure 2 may not appear to agree with illustrated trends in this figure. This is because of normalization of follicular waves for individual ewes to the mean day of wave emergence of each wave within the two groups in this figure. This figure is included to show the overall pattern of peaks in serum concentrations of FSH and follicular waves, in cycles with three or four follicular waves.

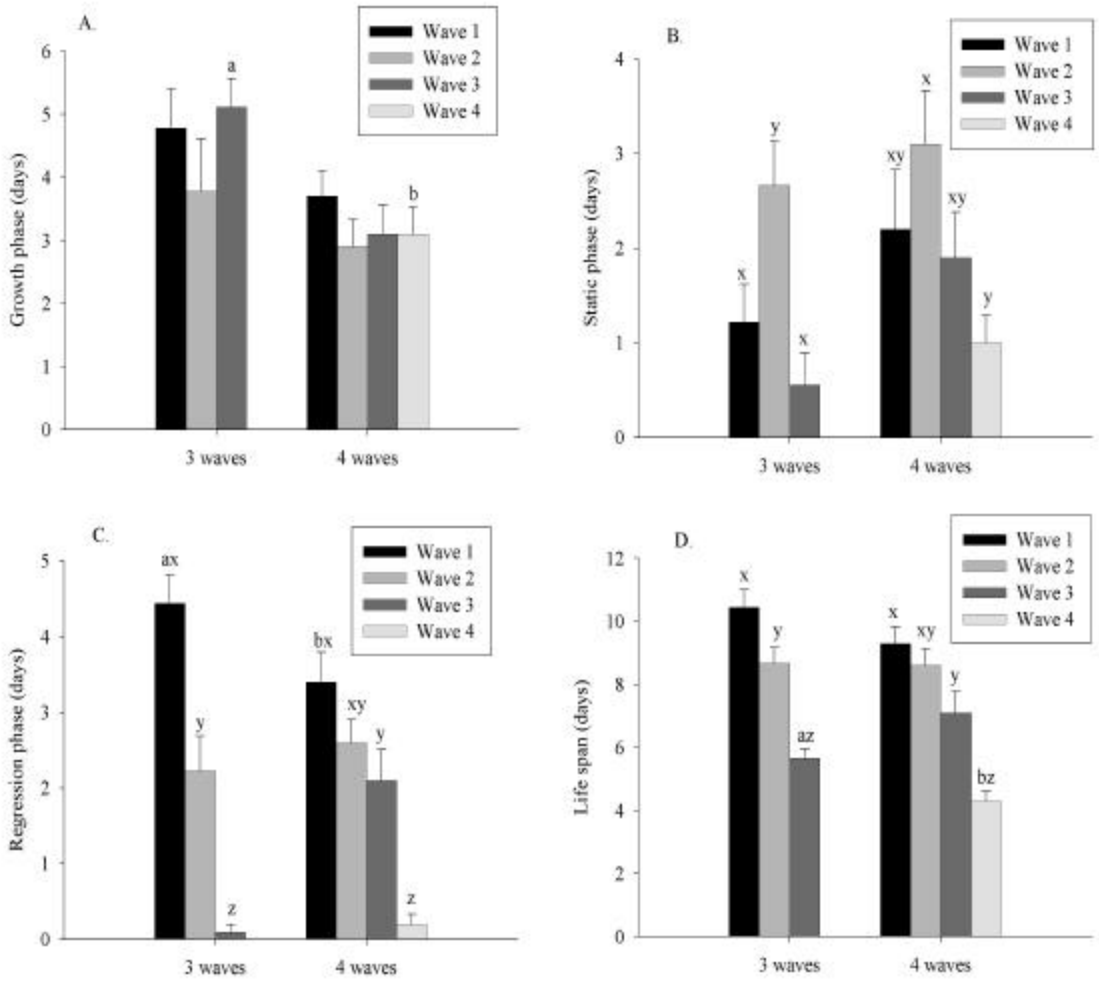


Fig. 2.2. The growth, static and regression phase and lifespan of the largest follicle growing in Wave 1 (black bars), Wave 2 (gray bars), Wave 3 (dark grey bars) and Wave 4 (light gray bars) in three- (n=9) or four- (n=10) wave cycles. Wave 1 and Wave 2 of the three-wave cycles were compared with respective waves of the four-wave cycles and Wave 3 (ovulatory wave) of the three-wave cycles was compared with Wave 4 (ovulatory wave) of the four-wave cycles. Letters (a-b) indicate differences between cycles with three waves and cycles with four waves per cycle ($P<0.05$) within the respective waves. Letters (x-y) indicate differences between the waves ($P<0.05$) within three-wave or four-wave cycles.

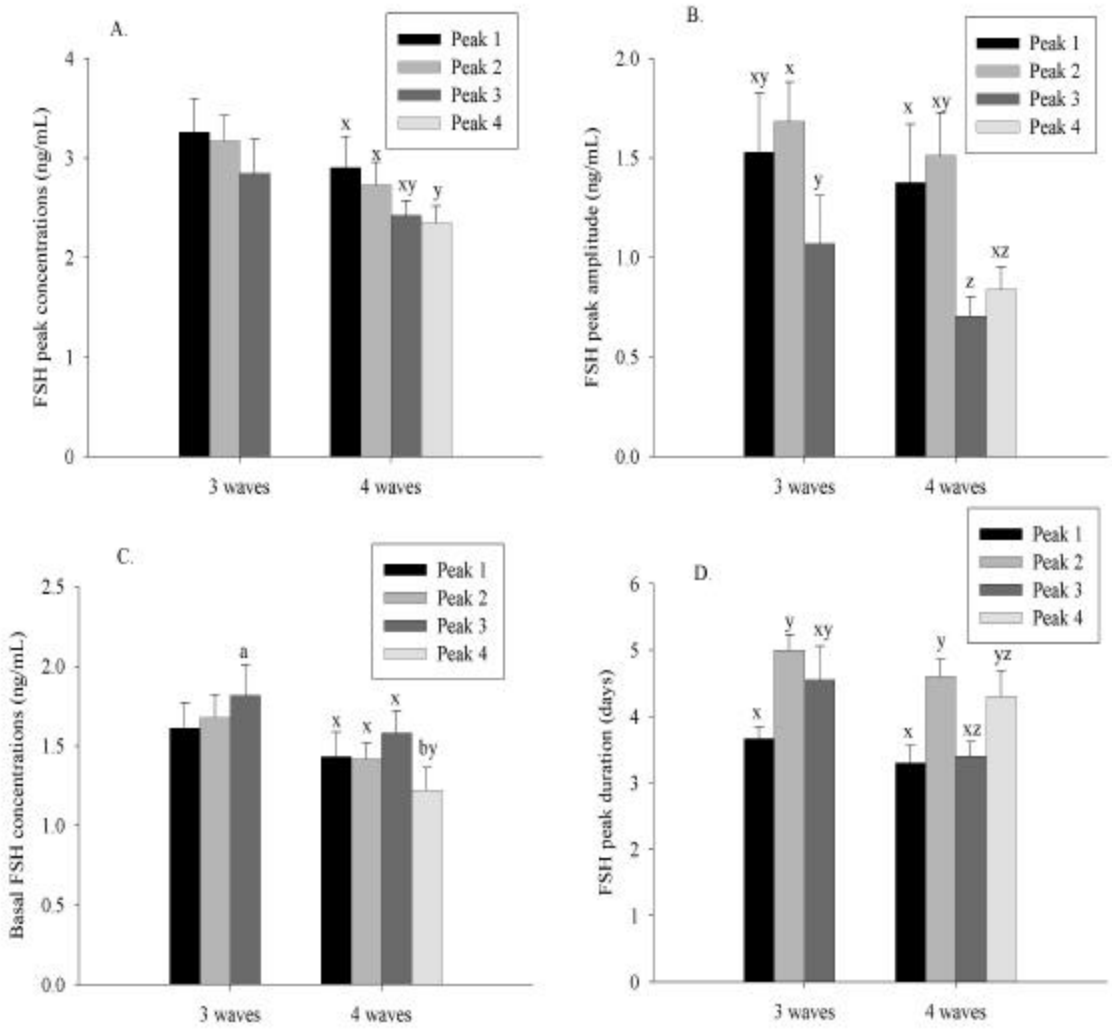


Fig. 2.3. Characteristics of serum FSH concentrations (FSH peak concentration, amplitude and duration and basal serum FSH concentrations; mean ± S.E.M) of FSH peak 1 (black bars), peak 2 (gray bars), peak 3 (dark grey bars) and peak 4 (light gray bars) in three (n=9) or four (n=10) wave cycles. FSH peak 1 and peak 2 of the three-wave cycles were compared with the respective peaks of the four-wave cycles and peak 3 (peak triggering the ovulatory wave) of three-wave cycles was compared with the Wave 4 (peak triggering the ovulatory wave) of the four-wave cycles. Letters (a-b) indicate differences between ewes with three waves and ewes with four waves per cycle ($P<0.05$) within the respective peaks. Letters (x-y) indicate differences between the peaks ($P<0.05$) within three-wave or four-wave cycles.

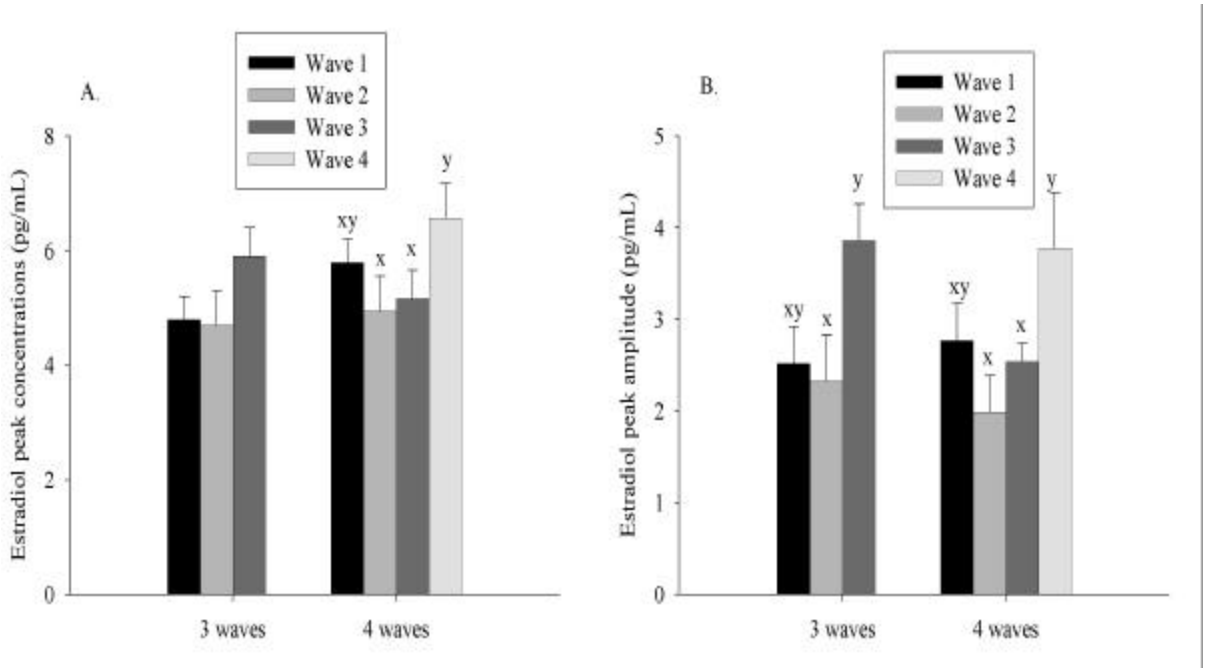


Fig. 2.4. Estradiol peak concentration and amplitude during wave 1 (black bars), wave 2 (gray bars), wave 3 (dark grey bars) and wave 4 (light gray bars) in three- (n=9) or four- (n=10) wave cycles. Wave 1 and Wave 2 of the three-wave cycles were compared with respective waves of the four-wave cycles and Wave 3 (ovulatory wave) of the three-wave cycles was compared with Wave 4 (ovulatory wave) of the four-wave cycles. Letters (x-y) indicate differences between the waves ($P<0.05$) within three-wave or four-wave cycles.

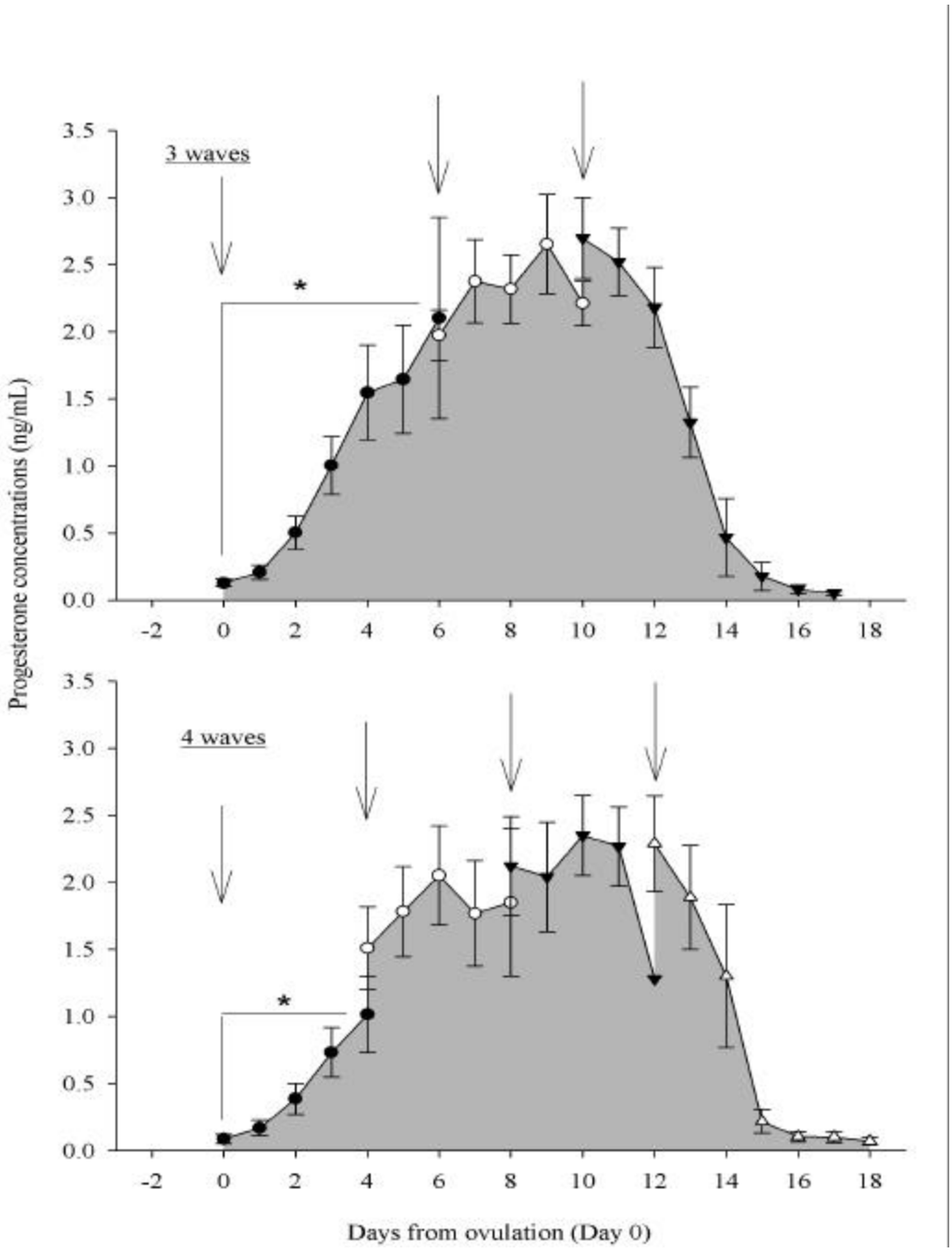


Fig. 2.5. Mean (±S.E.M.) daily serum progesterone concentrations (outlined with shading) in three- (top panel; n=9) or four- (bottom panel; n=10) wave cycles. Serum progesterone concentrations in the each IWI for all ewes are shown normalized to the respective mean day of wave emergence (indicated by the arrows).
Asterisks (*) indicate differences between three-wave and four-wave cycles with respect to IWI1 ($P<0.05$).

## 2.5 DISCUSSION

In the present study, the length of the estrous cycle was similar between cycles with three and four waves. This finding is of interest, as in cattle, estrous cycle length is longer for cows with three compared to two follicular waves per cycle (Celik et al., 2005; Ginther et al., 1989; Jaiswal et al., 2009; Noseir, 2003). However, in another study (Savio et al., 1988) in cattle, no significant difference in the length of the estrous cycle was seen with two or three waves per cycle. In a previous study in sheep it was shown that 8% of the ewes had three waves, 58% of the ewes had four waves and 34% of the ewes had five or six waves of follicular development during the estrous cycle (Ginther et al., 1995). In that study, three waves of follicular activity were recorded mainly during short estrous cycles (9-14 d), which occurred during the end of the breeding season and five or six waves occurred during cycles of abnormally prolonged lengths (22-24 d). In one other study (Bartlewski et al., 1999a), two ewes showed prolonged estrous cycle length (23 d) which was associated with four waves of follicular activity. The length of the estrous cycle in ewes in the breeding season is generally remarkably consistent (Goodman, 1994) and there is little effect of breed and age (Hafez, 1952). Therefore, the variation in numbers of follicular waves and length of estrous cycle in previous studies above could be partly due to abnormal estrous cycles, perhaps involving aberrant endocrine control. Interestingly, in a previous study in sheep (Nephew et al., 1991), when short cycles were compared with longer estrous cycles, it was found that serum progesterone concentrations were greater over days two to four of the cycle for shorter compared to longer cycles.

In the present study, the length of the lifespan and regression phase of the largest follicle of a wave decreased from the first to the last or ovulatory wave of the estrous cycle within three-wave and four-wave cycles. Further, although not significant in every case, FSH peak concentration and amplitude declined across the cycle. It is interesting that normal development of follicular waves occurs even with the decline in FSH peak concentrations and amplitude across the cycle. In previous studies performed in sheep (Bartlewski et al., 1999a) and in cattle (Ginther et al., 1989), the largest follicle growing in the first wave of the estrous cycle had a longer lifespan than follicles in other waves. In another study in sheep, the ovulatory follicle had a longer lifespan compared to follicles growing in other waves (Ravindra et al., 1994). The decrease in the lifespan and length of the regression phase across the cycle, noted in the present study, could be partly due to the decreased FSH peak concentration under progesterone dominance seen during the mid-luteal phase of the estrous cycle (Bartlewski et al., 1999a).

The IWI's were longer for the first and last waves of the cycle compared to the intervening intervals and were longer in cycles with three compared to four follicular waves. The IWI-1 could be longer due to a delay in the second FSH peak of the cycle by increasing serum progesterone concentrations at the onset of the cycle as the ewe is moving from a period of estrogen dominance into the luteal phase. Peaks in serum FSH concentrations were seen at regular intervals once the luteal phase was established. The long IWI at the end of the cycle was not reflected in any specific follicular wave characteristic but rather could reflect the timing of the ovulatory mechanism. The regression phase of the first follicular wave of the cycle was longer in three wave compared to four wave cycles as were the length of the lifespan and growth phase of the

last or ovulatory wave of the cycle. We did not see any consistent trends in hormone profiles to explain the difference in IWI between the two groups of cycles studied. Comparing inhibin concentrations in serum between cycles with three or four waves would be interesting. In cattle, it was shown that inhibin concentrations reached their peak in plasma sooner during the first wave of the cycle with two compared to three waves (Parker et al., 2003).

In the present study, the maximum follicle diameter of the largest follicle growing in the first wave and the last or ovulatory wave of the cycle was greater compared to the largest follicle growing in other waves in three-wave and four-wave cycles. There was no difference in maximum follicle diameter between three-wave and four-wave cycles within respective waves. This trend in maximum follicle diameter across the estrous cycle is similar to that seen in goats (Ginther and Kot, 1994) and cattle (Ginther et al., 1989). In two previous studies in sheep, only the largest follicle growing in Wave 1 (Ginther et al., 1995) or the ovulatory wave (Ravindra et al., 1994) attained a significantly greater maximum follicle diameter compared to larger follicles growing in other waves during the estrous cycle. However, in two other previous studies in sheep, it was found that follicles growing in the ovulatory and non-ovulatory waves of the cycle are similar in maximum follicle diameter (Bartlewski et al., 1999a; Souza et al., 1998). In cattle, the diameter of the ovulatory follicle was smaller in estrous cycles with three waves compared to two waves (Ginther et al., 1989). The greater maximum follicle diameter in Wave 1 and the last or ovulatory wave, compared to other waves, in the present study, could be because these follicles would have grown during the early and late luteal phase of the cycle when serum progesterone

concentrations were low (Leyva et al., 1998). Hence, these follicles would have been exposed to a greater frequency of LH pulses compared to follicles growing in the mid-luteal phase of the cycle (Baird, 1978; Martin, 1984; Rawlings and Cook, 1993; Wheaton et al., 1984). This inverse relationship between the progesterone and serum LH pulse frequency has been well documented (Baird, 1978; Martin, 1984; Rawlings and Cook 1993; Wheaton et al., 1984). Treatment of ewes with subluteal serum progesterone concentrations caused follicles to grow longer and to get larger and also decreased follicular turnover (Vinoles et al., 2001). It is interesting to note that in general, the numbers of large follicles (≥ 5 mm in diameter) were lowest for the first and last wave of the cycle in the present study. This same trend was roughly seen for 4 mm follicles as well, and we suggest that for the first and the last waves of the cycle, when exposed to a high frequency of LH pulses, fewer follicles enter waves but those follicles grew to a larger size than those seen in other waves in the cycle.

In the present study, the length of the static phase of the largest follicles growing in wave 2 of the cycle, was greater in both three-wave and four-wave cycles compared to other waves; however, this was not significant in comparison to Waves 1 and 3 in four-wave cycles. It was suggested that follicles growing in the mid-luteal phase have a shorter static phase because of the decreased gonadotropin support seen under progesterone dominance (Bartlewski et al., 1999a). However, the reason for the increased static phase in Wave 2 in the present study is not entirely clear.

The length of the growth phase of the largest follicle in waves appeared to be longer for all the waves in three-wave ewes compared to four-wave ewes, but significance was noted only for the ovulatory wave. The length of the growth phases

would appear to simply reflect the number of waves present in a cycle (i.e longer if there are only three waves per cycle). However, the reason for this is again unclear; follicles in three wave and four wave ewes did not differ in maximum follicle size or estradiol production.

Basal FSH concentrations in the present study were based on only the nadir values for FSH on either side of an FSH peak and would intuitively be expected to be less important for regulating follicular waves than peak serum FSH concentrations or amplitude. However, the similarities in the data trends for the length of the follicular growth phase and basal serum concentrations of FSH are noteworthy. The data obtained for FSH peak duration were quite variable; in agreement with previous observations for sheep (Bartlewski et al., 1999a).

Serum estradiol concentrations reach a peak coinciding with the end of the growth phase of the largest follicle of a follicular wave in the ewe (Bartlewski et al., 1999a; Souza et al., 1998). In the present study, peak serum estradiol concentrations were generally greater for the ovulatory wave compared to other waves as is seen in cattle (Noseir, 2003). No differences were seen between three- and four-wave ewes; again in agreement with observations for two- and three-wave cows (Ahmad et al., 1997; Celik et al., 2005).

In summary, the length of the estrous cycle was similar between cycles with three and four waves. The mechanism that makes a three-wave or four-wave cycle is unclear as there were remarkably few differences in FSH secretory patterns or characteristics of follicular waves between three- and four-wave cycles. However, the IWI was longer for the first and the ovulatory wave of the cycle in three- compared to

four-wave cycles. Estrous cycles with three waves had ovulatory waves with a longer growth phase and lifespan compared to cycles with four waves. Also, in those cycles with three waves, the regression phase for the first-wave of the cycle was longer compared to cycles with four-waves. The lifespan and the length of the regression phase of the largest follicle of a wave and, although not significant in every case, FSH peak concentration and amplitude generally declined across the estrous cycle. The maximum follicular diameter of the largest follicle growing in the first-wave and the ovulatory wave of the cycle was greater compared to other waves of the cycle. The IWI was longer for the first-wave and the ovulatory-wave of the cycle compared to other waves of the cycle.

To conclude, estrous cycles with three or four follicular waves were confined within the same length of cycle. In this study, there were no consistent endocrine or follicular characteristics that appeared to explain the number of waves per cycle. In fact, the clearest trends in the study were for endocrine and follicular characteristics to change across the cycle in a similar manner regardless of the number of waves per cycle.

# Chapter 3: PULSATILE LH SECRETION AND OVARIAN ANTRAL FOLLICULAR WAVE EMERGENCE AND GROWTH IN ANESTROUS EWES*

Seekallu SV, Toosi BM, Clarke K and Rawlings NC

## 3.1 ABSTRACT

In anestrous ewes, treatment with estradiol-releasing implants abolished pulsed LH secretion and blocked emergence of follicular waves; FSH secretion was only minimally affected. In the present study, pulsed LH secretion was restored by GnRH treatment in anestrous ewes treated as described above to see if restored pulsed LH secretion would restore follicular waves. Twelve ewes received subcutaneous silastic rubber implants (10 x 0.47 cm) containing 10% estradiol-17ß w/w for 12 d. Six ewes received GnRH injections (200ng; IV) every 4 h for the last 6 d that the implants were in place to reinitiate pulsed LH secretion. Control ewes (n=6) received saline. Ovarian ultrasonography and blood sampling was done daily; blood samples were also taken every 12 min for 6 h on Days 6 and 12 of the treatment period. Treatment with estradiol blocked pulsatile secretion of LH ($P<0.001$). Follicular waves were suppressed during treatment with estradiol, but resumed following the start of GnRH injection. The range of peaks in serum FSH concentrations that preceded and triggered follicular wave emergence were almost the same in ewes given estradiol implants alone or with GnRH; mean concentrations did not differ ($P<0.05$). We concluded that some level of pulsatile LH secretion is required for the emergence of follicular waves that are triggered by peaks in serum concentrations of FSH in the anestrous ewe.

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## 3.2 INTRODUCTION

With the recent use of transrectal ovarian ultrasonography, in both cyclic and anestrous ewes, it was found that 1 to 3 ovarian antral follicles emerge or grow from a pool of small follicles, 1-3 mm in diameter, every 4 to 5 d (Bartlewski et al., 1999a; Bartlewski et al., 1998; Schrick et al., 1993; Souza et al., 1996) These follicles grow to a diameter of = 5 mm in diameter before regression or ovulation (Bartlewski et al., 1999a; Bartlewski et al., 1998; Schrick et al., 1993; Souza et al., 1996). Each wave of follicle growth is preceded by a transient peak of FSH secretion that lasts 3 or 4 d and can be characterized by blood samples taken daily (Bartlewski et al., 1999a; Bartlewski et al., 2000c; Duggavathi et al., 2004). This peak in FSH secretion is an essential trigger for the follicular wave (Bartlewski et al., 1999a; Bartlewski et al., 2000c; Duggavathi et al., 2004).

Pulses of LH are released at a frequency of about 1 every 1 to 6 hrs in the ewe and last no more than about 2 hrs (Bartlewski et al., 2000a; Duggavathi et al., 2005b). Although peaks in FSH secretion initiate follicular waves, the role of pulsed LH secretion in the emergence, growth and regression of ovine antral follicles is unclear. Changes in LH pulse frequency during the estrous cycle do not appear to be correlated with or functionally related to specific phases of the growth or regression of follicular waves in the ewe (Bartlewski et al., 2000a; Duggavathi et al., 2005b). FSH secretory profiles in blood samples collected from the jugular vein in ewes are not pulsatile (Wallace and McNeilly, 1986; Wheaton et al., 1984).

Compared to ewes during the breeding season, seasonally anestrous ewes have lower circulating concentrations of LH, FSH, and estradiol, little or no progesterone and

serum concentrations of estradiol and inhibin are not correlated with follicular wave development (Bartlewski et al., 2000a; Evans et al, 2001a; McNatty et al., 1984b; Walton et al., 1980; Yuthasastrakosol et al., 1977) The frequency of LH secretory pulses is very low and fluctuates very little across seasonal anestrus (Jackson and Davis, 1979; McNatty et al., 1981; McNatty et al., 1984b; Walton et al., 1980; Yuthasastrakosol et al., 1977), affording no correlation to stages of follicular wave development and regression. During seasonal anestrus, estradiol exerts a more powerful negative feedback effect on pulsatile LH secretion than during the breeding season but the effects on FSH secretion appear to be minimal (Joseph et al., 1992; Karsch et al., 1987; Karsch and Foster, 1975; Karsch et al., 1983).

When cyclic ewes were treated for 10 d with large estradiol-releasing implants (10 x 0.47 cm), that created supra physiological serum concentrations of estradiol-17ß (10.4 ± 0.7 pg/mL vs 3.9 ± 0.7 pg/mL, treated vs control ewes, respectively), the amplitude of the FSH peaks that precede follicular waves was reduced and follicle wave emergence was blocked (Barrett et al., 2006). Injection of ovine FSH (oFSH), to recreate FSH peaks, re-initiated follicular waves (Barrett et al., 2006). In this study, pulsed LH secretion was not affected. In anestrous ewes (Barrett, 2007), large estradiol-releasing implants completely abolished LH pulsatility and blocked follicular wave emergence. Interestingly, in these ewes, FSH secretion was only minimally affected. Based on this study, it was concluded that although peaks in FSH secretion initiate follicular waves in the anestrous ewe, this also requires some level of pulsed LH secretion. The purpose of the present experiment was to replace LH pulses by using frequent GnRH injections in the experimental model above in anestrous ewes, to see if

restored LH pulsatility would infact allow restoration of follicular waves. This would confirm that although peaks in FSH secretion initiate follicular waves in the anestrous ewe, some level of pulsed LH secretion is also required.

We hypothesized that although the frequency of pulses of LH secretion is very low in anestrous ewes, pulsatile LH secretion is still essential for the emergence and growth of ovarian follicular waves. The objective of this study was to examine the need for pulsed LH secretion for the emergence and growth of ovarian follicular waves in anestrous ewes.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Animals

Care and handling of experimental animals was done according to the Canadian Council on Animal Cares' published guidelines. Sexually mature, clinically healthy, seasonally anestrous, WWF ewes were kept outdoors in sheltered paddocks. Ewes were fed a maintenance diet of hay; cobalt-iodized saltlicks and water were freely available. The WWF is a cross between the Columbia and Rambouillet breeds.

### 3.3.2 Ultrasound Technique

The growth and regression of ovarian antral follicles were monitored in all ewes by transrectal ovarian ultrasonography (scanning) using a 7.5-MHz linear transducer stiffened with a hollow plastic rod and connected to a B-mode, real-time echo camera (Aloka SSD-900, Overseas Monitor, Richmond, BC, Canada). This technique has been validated for monitoring ovarian follicular dynamics and CL detection in sheep (Duggavathi et al., 2003; Ravindra et al., 1994; Schrick et al., 1993). All images were viewed at a magnification of X 1.5 with constant gain and focal point settings. Ovarian

images were recorded (Panasonic AG 1978, Matsushita Electric, Mississauga, ON, Canada) on high-grade video tapes (Fuji S-VHS, ST-120 N, Fujifilm, Tokyo, Japan) for later examination. The relative position and dimension of follicles and luteal structures were also sketched on ovarian charts.

### 3.3.3 Experimental Design

Twelve seasonally anestrous (May/June) WWF ewes (mean body weight of 83.5 ± 3.0 kg) received subcutaneous silastic rubber implants (10 x 0.47 cm) containing 10% estradiol-17ß w/w for 12 d (95 mg/implant; Day 0 is the day of implant insertion; Figure 3.1). To make the implants, liquid silastic rubber (A-101 medical grade silicone elastomer; Factor II, Inc., Lakeside, AZ, USA) was mixed with the steroid and a curing catalyst was added (Catalyst; Factor II, Inc., Lakeside, AZ, USA). The mixture was injected into silastic tubing (Silastic laboratory grade tubing; 0.34 cm i.d. x 0.47 cm o.d.; Dow Corning, Midland, MI, USA) and, once cured, the tubing was cut into 10cm lengths. Implants were soaked in sterile 0.9% (w/v) saline for 36-48 h at room temperature before insertion to saturate the implant and prevent a sudden peak of release of estradiol when the implants were inserted into the ewes. Lidocaine hydrochloride (2%; Xylocaine; AstraZeneca Canada Inc., Mississauga, ON, CA) was used as a local anesthetic. A 1.5 cm incision was made in the axillary region with a scalpel, the implant was inserted using a trocar, and the incision was closed with wound clips (9 mm MikRon AUTOCLIP; Becton Dickinson Primary Care Diagnostics, Sparks, MD, USA). Implants were removed from all ewes 12 d after insertion (Day 0= day of insertion). Six of the twelve ewes were injected with GnRH (200ng; i.v; in saline; Sigma Chemical Company, St. Louis, MO, USA) every 4 h for the last 6 d that the

implants were in place, starting at 0800 h on Day 6 (Figure 3.1). The dose of GnRH was designed to create LH pulses within a physiological range. Six control ewes received only saline (IV). Daily scanning and blood sampling were done from 5 d before to 22 d after implant insertion. Blood samples were taken by jugular venipuncture (10 mL; Becton Dickinson, Franklin Lakes, NJ, USA) starting at 0800 h, followed immediately by scanning. Blood samples were also collected every 12 min, by a catheter inserted in the jugular vein, starting 36 min before the first and penultimate GnRH/ saline injections and ending 2 h after the second and last GnRH/ saline injections respectively, in order to characterize the secretory patterns of LH and FSH.

### 3.3.4 Analysis of follicular data

A follicular wave consisted of a follicle or a group of follicles that emerged and grew from 2 or 3 mm in diameter to =5mm (growth phase), before regressing to 2 or 3 mm in diameter (regression phase); time spent at =5mm was regarded as the static phase (Bartlewski et al., 1998). Data were analyzed for the number of follicular waves in each group of ewes. The number of small follicles (=1 to =3 mm in diameter) each day of the study were also analyzed. Ovulation was detected with ultrasonography as the collapse of a large follicle (=5 mm in diameter) that had been followed in its growth/static phase for several days. Follicular data were integrated for both ovaries of each ewe.

### 3.3.5 Hormone analysis

All blood samples were permitted to clot at room temperature for 18 to 24 h. Samples were then centrifuged for 10 min at 1500 x g, and serum was removed and kept at -20 $^{o}$C until assayed.

Progesterone (Rawlings et al., 1984), estradiol (Currie and Rawlings, 1989; Joseph et al., 1992), LH and FSH (Rawlings et al., 1988) concentrations were measured in serum samples by validated radioimmunoassay (RIA) procedures. The assay sensitivities (defined as the lowest concentration of a hormone capable of significantly displacing radio-labeled hormone from the antibody) were: 0.03 ng/mL for progesterone, 1.0 pg/mL for estradiol and 0.1 ng/mL for FSH and LH. The ranges of standards were: 0.1 to 10 ng/mL, 1.0 to 100 pg/mL, 0.12 to 16.0 ng/mL, and 0.06 to 8.0 ng/mL in the progesterone, estradiol, FSH and LH assays, respectively. A concentration equivalent to the sensitivity of the assay was assigned to serum samples with hormone concentrations lower than the assay sensitivity. Serum samples collected daily, throughout the experimental period, were analyzed for concentrations of progesterone, estradiol, and FSH. All serum samples collected every 12 min were analyzed for concentrations of LH and FSH.

The intra- and inter-assay coefficients of variation (CVs) were 15.9% and 6.5% or 7.2% and 12.8% for reference sera with mean progesterone concentrations of 0.21 or 1.13 ng/mL, respectively. The intra- and inter-assay coefficients of variation were 7.4% and 9.0% or 5.5% and 6.8% for reference sera with mean estradiol concentrations of 7.97 or 25.17 pg/mL, respectively. The intra-assay CVs were 4.2% or 4.2% for reference sera with mean FSH concentrations of 1.12 or 3.34 ng/mL, respectively. The intra- and inter-assay CVs were 4.7% and 5.4% or 9.6% and 10.0% for reference sera with mean LH concentrations of 0.4 or 2.97 ng/mL, respectively.

The PC-PULSAR program (Gitzen and Ramirez, 1988) was used to assess mean serum LH and FSH concentrations as well as LH and FSH pulse frequency and amplitude in blood samples collected every 12 min for 6 h.

Peaks of FSH in blood samples taken daily were identified using cycle-detection software (Clifton and Steiner, 1983). A fluctuation or cycle was defined as a progressive rise and fall in hormone concentrations that encapsulated a peak concentration (nadir-to-peak-to-nadir; Clifton and Steiner, 1983). Follicle stimulating hormone peak concentration was defined as the concentration of FSH observed at the apex of the FSH peak.

### 3.3.6 Statistical analysis

Observations on hormone concentrations and ovarian follicles measured daily were normalized to the day of implant insertion for analysis and presentation. Two-way repeated measures ANOVA (Sigma Stat 7 for Windows Version 2.03, 1997, SPSS Inc., Chicago, IL, USA) was used to assess differences in hormone concentrations and observations on ovarian follicles over time and among the groups of ewes (i.e., treated with or without exogenous GnRH injection). Two-way repeated measures ANOVA was also used to assess differences in LH and FSH secretory characteristics from blood samples collected every 12 min for 6 h (i.e., mean concentrations, pulse frequency, and pulse amplitude) among groups of ewes and between intensive sampling days. If the main effects, or their interactions, were significant ($P<0.05$), Fisher's protected least significant difference (LSD) was used as a post-ANOVA test to detect differences between individual means ($P<0.05$). The Chi-square test was used to analyze for the proportion of missing FSH peaks. Data are expressed as mean ± S.E.M.

## 3.4 RESULTS

### 3.4.1 Mean daily estradiol- 17ß concentrations

Mean serum estradiol concentrations were greater during the implant treatment period (12.08 ± 0.7 and 10.81 ± 0.7 pg/mL) compared to the period before implant insertion (4.74 ± 0.6 and 3.63 ± 0.9 pg/mL) or after implant removal (4.33 ± 0.3 and 2.71 ± 0.2 pg/mL) in both control ewes (ewes treated with only estradiol implants) and ewes treated with GnRH, respectively (Figure 3.2).

### 3.4.2 Characteristics of serum LH concentrations

LH pulses were absent on Day 6 and on Day 12 after implant insertion, whereas in the ewes treated with GnRH, each GnRH injection led to a pulse in LH secretion (Figures 3.3C and 3.3D).

### 3.4.3 Characteristics of serum FSH concentrations

No difference was found in mean serum FSH concentrations amongst days of intensive blood sampling (Day 6 or 12) for control ewes or ewes treated with GnRH ($P>0.05$; Figure 3.3A and 3.3B). Within days of intensive sampling mean serum FSH concentrations did not differ between ewes treated with GnRH or the control ewes ($P>0.05$), but there was a time effect and an interaction of treatment by time ($P<0.001$; Figure 3.3A and 3.3B). Comparison of individual means showed that serum FSH concentrations were significantly greater in ewes given GnRH compared to control ewes from 12 min to 60 min after the first GnRH injection and from 24 to 36 min after the second GnRH injection during the second intensive sampling period (Day 12; $P<0.05$; Figure 3.3B).

Based on blood samples taken daily, the peak serum FSH concentrations on Day 10 were significantly less than that seen on Day 4 of the treatment period ($P<0.05$; Figure 3.4). In Figure 3.4, in ewes given only estradiol implants, peak serum concentrations during treatment and where follicular waves were blocked, had a range of 0.7 to 2.1 ng/mL (mean of 1.3 ± 0.17 ng/mL). In ewes given GnRH, FSH peaks occurring at a similar time and that were accompanied by wave emergence, covered a range of 0.8 to 2.4 ng/mL (mean of 1.5 ± 0.17 ng/mL). The mean peak concentrations did not differ between control ewes and ewes treated with GnRH ($P>0.05$).

One control ewe and 3 ewes given GnRH did not have a detectable increase in FSH at the time of the 2$^{nd}$ FSH peak seen for these groups and 2 control ewes and 1 ewe given GnRH did not have a detectable increase in FSH at the time of the 4$^{th}$ FSH peak seen for these groups. However, the results of Chi square analysis comparing the proportion of missed FSH peaks between groups was not significant ($P>0.05$).

### 3.4.4 Antral Follicle Development

All control ewes given only large estradiol releasing implants had no follicle waves emerging during the implant treatment period (Figure 3.4). In ewes treated with GnRH, four ewes had follicle wave emergence on Day 5 and five ewes on Day 8 during the implant treatment period. All ewes showed normal follicular waves before implant insertion and after implant removal.

Over the implant treatment period, the number of small follicles did not differ between control ewes (26.48 ± 2.3) and ewes treated with GnRH (25.13 ± 1.7) ($P>0.05$).

### 3.4.5 Cystic follicles and mean daily serum progesterone concentrations

We saw development of cystic follicles or luteinized follicles in the present study, when ewes were treated with estradiol. Three ewes from each of the treatment groups formed a cystic follicle 3 d after implant insertion.

Mean serum progesterone concentrations were significantly higher in ewes that had cystic follicles compared to ewes that had no cystic follicles respectively (0.21 ± 0.05 ng/mL vs 0.08 ± 0.05 ng/mL; $P<0.05$). Serum progesterone concentrations increased significantly to a peak of 0.44 ± 0.10 ng/mL on Day 9 after implant insertion in those ewes that had cystic follicles (n=6).

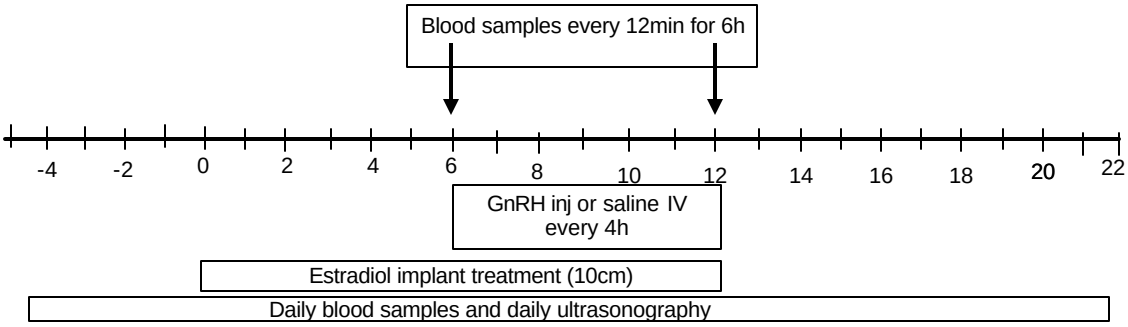


Fig. 3. 1. Schematic representation of the experimental design Estradiol implants were inserted subcutaneously on Day 0 and left in place for 12d. Injections of GnRH (200ng; IV) or saline (IV) were given every 4h from Day 6 to Day 12 after implant insertion. Daily ultrasound scanning and blood sampling was done from 5 d before to 22 d after implant insertion. Blood samples were also taken every 12min for 6h (intensive bleeds) on Day 6 and Day 12 to characterize the secretory patterns of LH and FSH. Intensive bleeds started 36 min prior to the first and penultimate injection of GnRH or saline and ended 2 hrs after the second and last injections respectively.

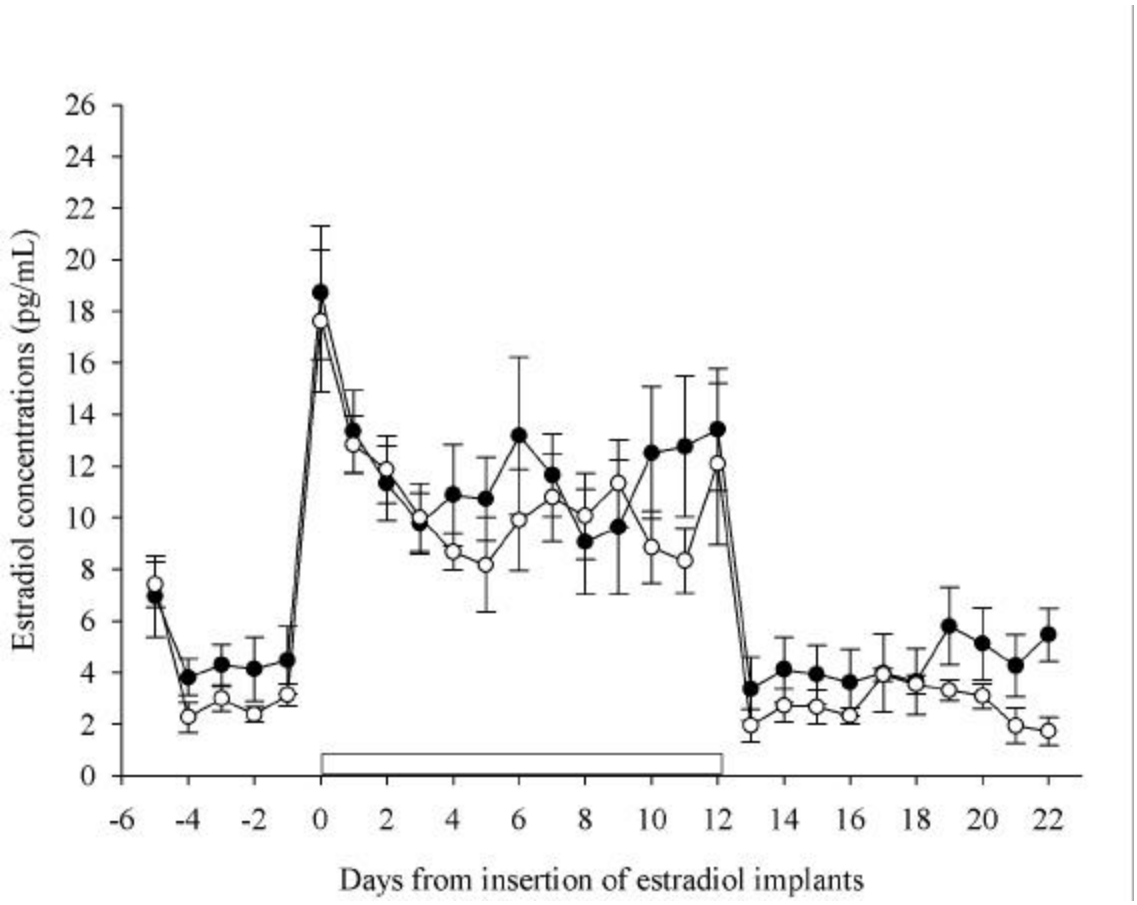


Fig. 3. 2. Mean (±S.E.M.) daily serum estradiol concentrations in anestrous Western White Face ewes treated for 12 d (Day 0-12; open rectangle on X-axis) with large silastic rubber implants containing 10% estradiol-17ß (s.c 10 x 0.47 cm) with (open circles; n=6) or without (black circles; n=6) GnRH treatment for 6 d, starting on day 6 of implant insertion. Data were normalized to the day of implant insertion (Day 0) in all ewes.

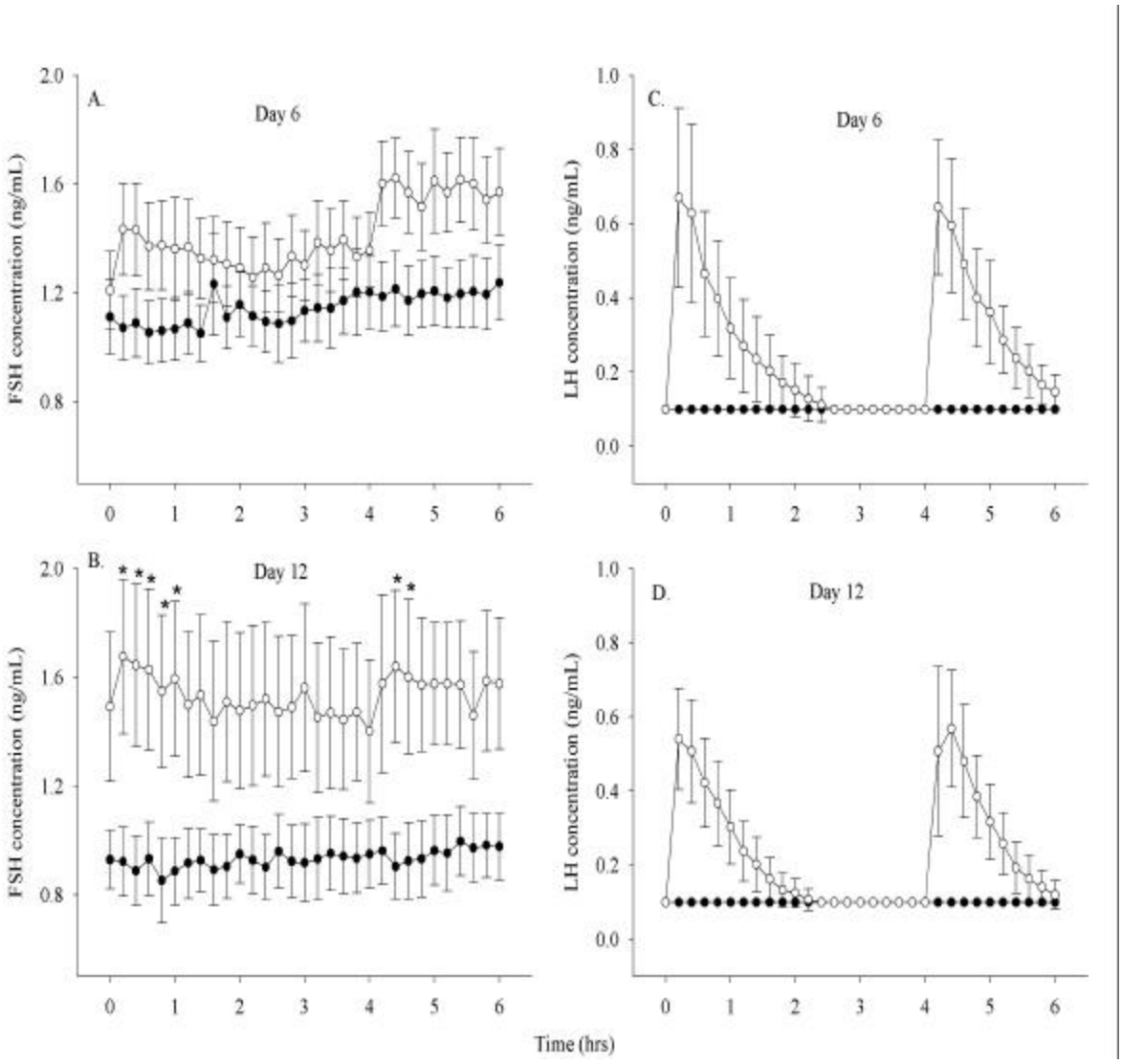


Fig. 3. 3. Mean (±S.E.M.) serum FSH concentration and mean (∀S.E.M.) serum LH concentrations determined from serum samples collected every 12 min for 6 h on Day 6 (panel A and C, respectively) and Day 12 (panel B and D, respectively) after implant insertion in anestrous Western White Face ewes treated for 12 d with large silastic rubber implants containing 10% estradiol-17ß (s.c 10 x 0.47 cm) with (open circles; n=6) or without (black circles; n=6) GnRH treatment every 4 h for 6 d, starting on day 6 after implant insertion. Note that LH concentrations in all the samples in control ewes were below the assay sensitivity. They were assigned a concentration equivalent to the sensitivity of the assay (0.1ng/mL; Panel C and D). * P<0.05 between the groups within respective bleeding points.

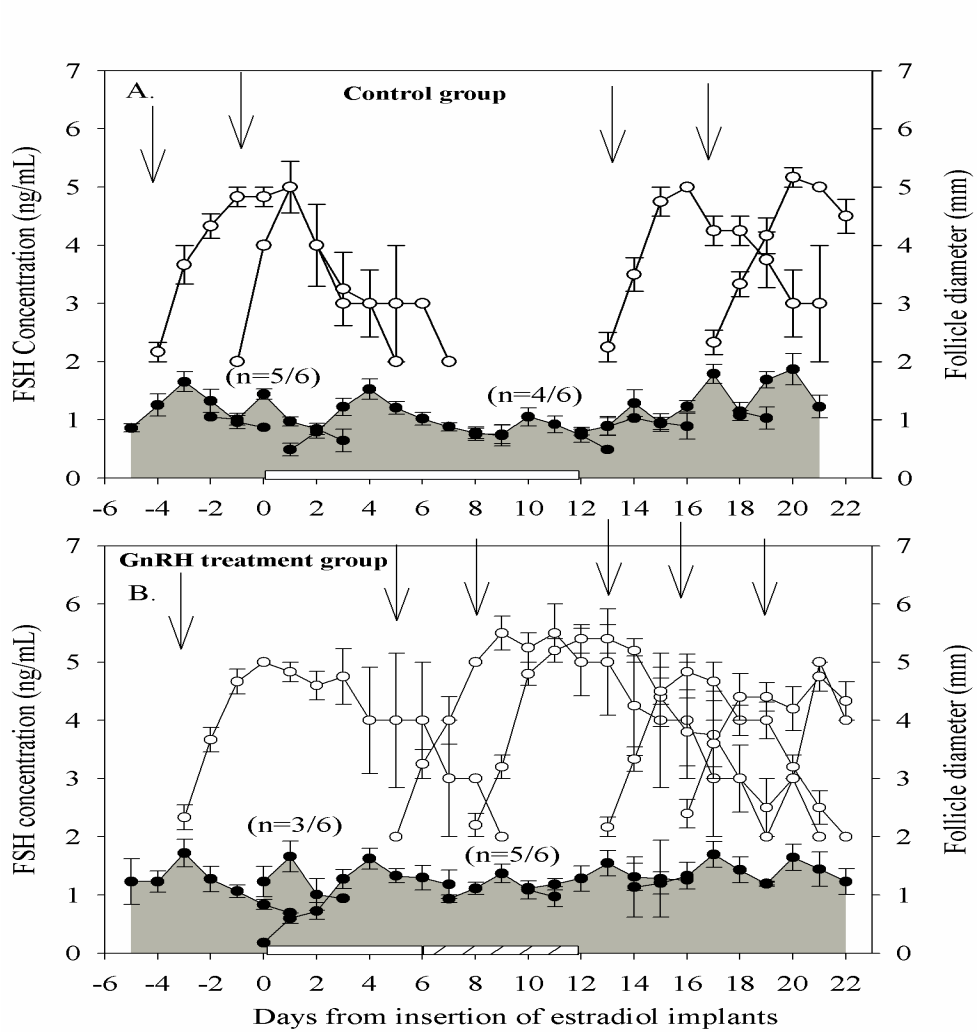


Fig. 3.4. Peaks in serum concentrations of FSH (outlined with shading) and their associated emerging follicle waves (open circles) in anestrous Western White Face ewes treated for 12 d (Day 0-12; open rectangle on X-axis) with large silastic rubber implants containing 10% estradiol-17ß (s.c 10 x 0.47 cm) without (panel A; n=6) or with (panel B; n=6) GnRH treatment for 6 d (Day 6-12; checked rectangle on X-axis), starting on day 6 of implant insertion (200ng; IV; every 4 hrs for 6 d). Data were normalized to the day of implant insertion (Day 0) in all ewes. The average curves representing the growth, static and regression phases of all ewes in a group were normalized for each follicle wave to the mean day of wave emergence (indicated by the arrow; [Barrett et al., 2006]). All FSH peaks for all ewes in a group are shown normalized to the mean day of occurrence of the apex of the FSH peak for each wave. For every FSH peak, serum concentration profiles were delimited by the encompassing nadirs of the FSH concentrations (hence the overlap of the data for adjacent peaks in some cases). In the control group, a follicular wave emerged and grew in only one ewe the day before implant insertion (indicated by †). Numbers in parenthesis give the number of ewes in a group showing an FSH peak at that time. Concentrations of FSH and follicle diameters are expressed as mean ∀S.E.M.

## 3.5 DISCUSSION

Previously, in anestrous ewes, it was shown that supra-physiological concentrations of estradiol suppressed follicular wave development by abolishing pulsed LH secretion (Barrett, 2007). However, in the latter study, FSH secretion was minimally affected. In the present study, the most profound effects of the estradiol implant treatments were to block follicular wave emergence and the pulsatile secretion of LH as in the previous study described above.

In the present study, there was some indication that the large implants caused suppression of FSH secretion, particularly toward the end of the period of implant treatment. Restoration of LH pulses, by the GnRH treatment, reinitiated follicular wave emergence. Even though GnRH injections transiently increased serum FSH concentrations up to 1 hr, as noted in blood samples collected every 12 min for 6 hrs, this increase was not enough to cause changes in peak serum FSH concentrations measured daily over the GnRH treatment period, in comparison to control ewes. The dose of GnRH was very low and designed to cause physiological pulses of LH. These findings strongly support our hypothesis that in the anestrous ewe the presence of pulsed LH secretion is required for the generation of the follicular waves that are triggered by peaks of FSH secretion.

In the present study, the FSH peaks, that were able to initiate follicular waves in the presence of restored pulsatile secretion of LH, were almost in the same range of concentration as those peaks occurring in control ewes at a similar time and where follicular waves were blocked during the period of implant treatment and absence of pulsed LH secretion. It is particularly intriguing that the peaks in serum concentrations

of FSH at Day 4 in GnRH-treated ewes gave rise to follicular waves on Day 5; even though pulsatile LH (GnRH) support was not restored until Day 6. With the ultrasound equipment utilized, we can track follicular waves back to their emergence from the pool of small follicles, 2-3 mm in diameter. The size of this pool of follicles was not affected by treatment with estradiol 17-ß. In the waves mentioned above, the transition to 4mm occurred on Days 6-7 during GnRH treatment. Follicular waves emerge on the day of the zenith of the peak in serum FSH concentration or one day before or after (Bartlewski et al., 1999a; Bartlewski et al., 2000c; Duggavathi et al., 2004). It should be noted that the peak concentration of FSH on Day 4 in control and GnRH treated ewes in the present study were virtually identical but the FSH peaks on Day 4 were only followed by follicular waves in the GnRH treated ewes.

In another recent study, the final growth of antral follicles destined to luteinize following prostaglandin and hCG treatment of ewes with ovarian autotransplants, did not appear to require pulsatile LH secretion (Campbell et al., 2007). In the latter study, LH secretion was suppressed by treatment with a GnRH antagonist. Final antral follicle growth appeared to have occurred in ewes given constant or pulsed infusion of LH and also in ewes receiving no supplemental LH. However, in the latter study, the growth of follicles during treatment was only significant at the higher of two doses of LH given by constant infusion. All ewes formed CL in response to prostaglandin and hCG treatment. In the present study, removal of pulsed LH secretion, failed to allow emergence of follicular waves but restoration of LH pulses by GnRH treatment allowed follicular waves to occur in response to FSH peaks.

Another interesting observation from the present study is that FSH peaks continued to occur in the absence of follicle wave development. If changes in the temporal pattern of secretion of products from follicular waves were important for regulating FSH peaks, based on the present results, estradiol could not be the hormone responsible as relatively constant serum concentrations resulted from the implant treatment. Inhibin also suppresses FSH secretion in the ewe; however, the specific role of inhibin in regulating FSH peaks has not been studied (Mann et al., 1992b). FSH peaks and the associated follicular waves are not regulated by inhibin in anestrous ewes as serum concentrations of inhibin do not change with the pattern of follicular waves in the non-cycling ewe (Evans et al, 2001a). As no follicular waves emerged during the treatment period, in control ewes of the present study, it is unlikely that secretory products of the follicles of a wave, could have entrained the second, third and fourth FSH peaks observed during the period of implant treatment. We can speculate that ovarian follicular feedback may not regulate the rhythm of the FSH peaks that precede follicle wave emergence in the ewe. In ovariectomized ewes FSH peaks are seen with a rhythm similar to the intact ewe (Duggavathi et al., 2005a).

It is intriguing that in our previous study in cyclic ewes (Barrett et al., 2006), when similar sized estradiol implants were used, supra-physiological concentrations of estradiol had no significant effect on the LH secretory pattern. However, in cyclic ewes, estradiol implants producing higher concentrations of estradiol did abolish pulsed LH secretion (Barrett et al., 2007). It has been shown previously that estradiol exerts a stronger negative feedback effect on LH secretion during seasonal anestrous compared

to the breeding season (Goodman et al., 1981a; Joseph et al., 1992; Karsch et al., 1980; Martin et al., 1983).

The progesterone produced by the cystic follicles or luteinized follicles, was very low compared to peak serum concentrations of progesterone during the normal luteal phase of the cyclic ewes (3.58 ± 0.18 ng/mL; (Bartlewski et al., 1999b)). We saw no evidence that the presence of cystic follicles affected the pattern of emergence and growth of follicular waves in response to GnRH.

Based on the present and previous related study by Barrett (2007), we concluded that the supra-physiological concentrations of estradiol-17β created by estradiol-releasing implants suppressed follicle wave development by abolishing LH pulses in anestrous ewes. Restoration of LH pulses in implant treated ewes, by frequent injections of GnRH, allowed the reestablishment of follicular waves that are stimulated by peaks in secretion of FSH. We clearly showed that LH pulses in the anestrous ewe are critical for the development of follicular waves. It is unclear whether secretory products from the follicles of a wave entrain the FSH peaks that initiate follicular waves in anestrous ewes.

# Chapter 4: LH PULSE FREQUENCY AND THE EMERGENCE AND GROWTH OF OVARIAN ANTRAL FOLLICULAR WAVES IN THE EWE DURING THE LUTEAL PHASE OF THE ESTROUS CYCLE [*]

Seekallu SV, Toosi BM and Rawlings NC

## 4.1 ABSTRACT

In the ewe, ovarian antral follicles emerge or grow from a pool of 2-3 mm follicles in a wave-like pattern, reaching =5mm in diameter before regression or ovulation. There are 3 or 4 such follicular waves during each estrous cycle. Each wave is preceded by a peak in serum FSH concentrations. The role of pulsatile LH in ovarian antral follicular emergence and growth is unclear; therefore, the purpose of the present study was to further define this role. Ewes (n=7) were given 200ng of GnRH (IV) every  h for 96 h from Day 7 of the estrous cycle, to increase LH pulse frequency. Controls (n=6) received saline. In a second study, ewes (n=6) received subcutaneous progesterone-releasing implants for 10 d starting on Day 4 of the cycle, to decrease LH pulse frequency. Controls (n=6) underwent sham surgery. Daily transrectal ovarian ultrasonography and blood sampling was performed on all ewes from the day of estrus to the day of ovulation at the end of the cycle of the study. At appropriate times, additional blood samples were taken every 12 min for 6 h and 36 min  for 6 h in studies 1 and 2, respectively. The largest follicle of the follicular wave growing when GnRH treatment started, grew to a larger diameter than the equivalent wave in control ewes ($P<0.05$). Mean serum estradiol and progesterone concentrations were higher but mean serum FSH concentrations were lower during GnRH treatment compared to control ewes ($P<0.05$). The increased serum concentrations of estradiol and progesterone, in GnRH treated ewes, suppressed a peak in serum concentrations of FSH, causing a

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follicular wave to be missed. Treatment with progesterone decreased the frequency of LH pulses but did not have any influence on serum FSH concentrations or follicular waves. We concluded that waves of ovarian follicular growth can occur at LH pulse frequencies lower than those seen in the luteal phase of the estrous cycle but frequencies seen in the follicular phase, when applied during the mid-luteal phase, in the presence of progesterone, do enhance follicular growth to resemble an ovulatory follicle, blocking the emrgence of the next wave.

## 4.2 INTRODUCTION

In the ewe, recent studies using transrectal ovarian ultrasonography have revealed a wave-like pattern in the growth and regression of ovarian antral follicles. During the breeding season (Bartlewski et al., 1999a; Evans et al., 2000; Ginther et al., 1995; Schrick et al., 1993) and anestrus (Bartlewski et al., 1998; Souza et al., 1996), 1-3 follicles emerge or continue to grow from a pool of small follicles (2-3 mm in diameter) every 3-5 d. These follicles reach diameters of = 5 mm before ovulation or regression. Each follicular wave is preceded by a peak in serum concentrations of FSH lasting 3 to 4 d; this peak has been shown to be an essential trigger for the emergence of a follicular wave (Bartlewski et al., 1999a; Bartlewski et al., 1998; Duggavathi et al., 2003; Evans et al., 2000; Ginther et al., 1995). Prior to the use of ultrasonography for repetitive, non invasive monitoring of ovarian structures in the ewe, studies on the endocrine regulation of ovarian folliculogenesis were largely limited to single surgical or post mortem observation of the ovary. From such studies, it was concluded that the growth of ovarian antral follicles beyond 2 to 3 mm in diameter was largely dependent on FSH, with the final growth and maturation becoming LH dependent (Campbell et al.,

1995; McNeilly et al., 1991a; Picton et al., 1990b; Scaramuzzi et al., 1993).

During the luteal phase of the estrous cycle in sheep, the frequency of secretion of pulses of LH is quite low, in the order of 1 to 2 pulses per 6 h (Goodman et al., 1981a). This low frequency is maintained by the negative feedback effects of progesterone in concert with estradiol (Goodman and Karsch, 1980; Karsch et al., 1979; Rawlings et al., 1984). In the follicular phase, LH pulse frequency increases to = 1 pulse per h, once the inhibitory effects of progesterone are removed (Baird, 1978; Karsch et al., 1980). The reduced LH pulse frequency of the luteal phase is believed to hold final follicular growth and development in check while the enhanced pulse frequency of the follicular phase is believed to drive such maturation. In a recent study, using ewes with ovarian transplants and given a GnRH antagonist, antral follicles grew from 4.5 mm to 4.9 mm in diameter, over a period of 66 h with only basal LH secretion. This change in size was not significant and was not enhanced by treatment with pulses of LH or constant infusion of a low dose of LH over a similar time period. However, increasing the dose of LH given by constant infusion gave a significant increase in follicle size (3.9 to 5.0 mm in diameter) (Campbell et al., 2007). These follicles luteinized but did not ovulate in response to hCG. When we looked for correlation between characteristics of follicular waves (eg; length of the growth phase, maximum follicle size, numbers of follicles in a wave etc) at various time points in the estrous cycle and the secretory patterns of LH at the same time points, there was no indication of significant associations (Bartlewski et al., 2000a; Duggavathi et al., 2005b). However, when ewes were treated to create low serum progesterone concentrations from Day 4 after ovulation, LH pulse frequency increased and the largest follicles of the first wave of the

cycle was still growing 9 d later (Vinoles et al., 1999).

To try and clearly define the role of pulsatile LH secretion in the regulation of follicular waves in the ewe, we performed two experiments designed to alter LH pulse frequency over a wide range without affecting the FSH peaks that herald follicular waves. The objective of experiment 1 was to increase LH pulse frequency, during the midluteal phase of a cycle, to that seen in the follicular phase and the objective of experiment 2 was to suppress LH pulse frequency below that seen in the normal luteal phase. We hypothesized that altering LH pulse frequency over the range described would not influence any aspect of ovarian follicular waves.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Animals

Care and handling of experimental animals was done according to the Canadian Council on Animal Care's published guidelines. Sexually mature, clinically healthy, cycling (November-December), Western White Face (WWF) ewes were kept outdoors in sheltered paddocks (Saskatoon, SK, Canada; latitude: 52°10'N). Ewes were fed a maintenance diet of hay; cobalt-iodized saltlicks and water were freely available. The WWF is a cross between the Columbia and Rambouillet breeds. Ewes were monitored daily for estrus with vasectomized crayon-marker-harnessed rams.

### 4.3.2 Ultrasound Technique

Ovarian antral follicular dynamics were monitored in all ewes by transrectal ovarian ultrasonography (scanning) using a 7.5-MHz transducer stiffened with a hollow plastic rod and connected to a B-mode, real-time echo camera (Aloka SSD-900, Overseas Monitor, Richmond, BC, Canada). This technique has been validated for monitoring

ovarian follicular dynamics and for CL detection in sheep (Bartlewski et al., 1998; Duggavathi et al., 2003). All images were viewed at a magnification of X 1.5 with constant gain and focal point settings. Ovarian images were recorded (Panasonic AG 1978, Matsushita Electric, Mississauga, ON, Canada) on high-grade video tapes (Fuji S-VHS, ST-120 N, Fujifilm, Tokyo, Japan) for later examination. The relative position and dimension of follicles and luteal structures were also sketched on ovarian charts.

**4.3.3. Blood sampling**

Blood samples (10 mL) taken daily were collected by jugular venipuncture into Vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA). Intensive blood sampling and injection of GnRH or saline was done via indwelling jugular catheters (5 ml/sample; vinyl tubing, 1.0 mm inside diameter x 1.5 mm outside diameter; SV70, Critchley Electrical Products Pty Ltd., Auburn, NSW, Australia). All samples were permitted to clot at room temperature for 18 to 24 h. Samples were then centrifuged for 10 min at 1500 x g, and serum was removed and kept at -20 $^{o}$C until assayed.

**4.3.4 Experimental Design**

**4.3.4.1 Experiment 1**

Daily transrectal ovarian ultrasonography and blood sampling was performed on all 13 randomly selected ewes (mean body weight of 82.73 ± 2.5 kg), starting from the day when they were initially marked by the vasectomized crayon-marker-harnessed rams (estrus) until ovulation at the end of the cycle of study. Blood samples were taken daily at 12 noon, following ultrasound scanning. Starting at 8AM on Day 7 after ovulation (Day 0), seven ewes were given injections of GnRH (200ng; IV; in saline; Sigma Chemical Company, St. Louis, MO, USA; Figure 4.1) every h for 96 h, with the last injection given at 8AM on Day 11. The dose of GnRH was designed to create LH pulses that

were within a physiological range and was derived from dose trial studies. Six control ewes received injections of saline. Additional blood samples were taken every 12 min (intensive sampling), from 36 min before to 6 h after the GnRH injection given at 8AM on Day 7, Day 9 and Day 11 after ovulation.

#### 4.3.4.2 Experiment 2

Twelve randomly selected ewes (mean body weight of 79.04 ± 2.2 kg) were divided into two groups of six ewes each. Six ewes received subcutaneous silastic rubber implants (11x 0.48 cm; 2 implants per ewe) containing 10% progesterone w/w (125mg/implant; Sigma-Aldrich, Oakville, ON, Canada; Barrett et al., 2007) on Day 4 after ovulation (Day 0; Figure 4.1); implants remained in place for 10 d. Six sham operated control ewes received no implants. To make the implants, liquid silastic rubber (A-101 medical grade silicone elastomer; Factor II Inc., Lakeside, AZ) was mixed with the steroid, and a curing catalyst was added (Catalyst; Factor II Inc.). The mixture was injected into Tygon tubing moulds (0.48 cm i.d.; Norton Plastics, Akron, OH). Once cured, the progesterone implants were removed from the moulds, and cut into 11cm lengths. Implants were soaked in sterile saline, in a water bath, at 37°C for 48 h before insertion. Lidocaine hydrochloride (2%; Xylocaine; Astra Zeneca Canada Inc., Mississauga, ON, Canada) was used as a local anesthetic. A 1.5-cm incision was made in the axillary region with a scalpel, the implant was inserted using a trocar, and the incision was closed with wound clips (9-mm MikRon AUTOCLIP; Becton Dickinson Primary Care Diagnostics, Sparks, MD, USA). Each animal received one 11 cm implant in the left and right axillary regions, respectively. Daily transrectal ovarian ultrasonography and blood sampling was performed on the same schedule as described

for experiment 1. Blood samples were also taken every 12 min for 6 h on Day 3, Day 10 and Day 16 after ovulation starting at 8AM.

### 4.3.5 Follicular Data Analyses

A follicular wave consisted of a follicle or a group of follicles that emerged and grew from 2 or 3 mm in diameter to =5 mm (growth phase), before regressing to 2 or 3 mm in diameter (regression phase) or ovulation; time spent at =5mm was regarded as the static phase (Bartlewski et al., 1999a; Bartlewski et al., 1998). The length of the growth, static and regression phases, growth rate and life span of the largest follicle growing in follicular waves were analyzed for the interovulatory interval studied. The lifespan of a large antral follicle is defined as the interval from its emergence at 2 or 3mm to its regression back to 2 or 3mm. If more than 1 follicle attained the same maximum size, the follicle that reached the maximum diameter first and/or remained at its maximum size for the longest period of time, was regarded as the largest follicle of the wave. The number of small (=1 to =3 mm in diameter), medium (4mm in diameter), and large sized follicles (= 5mm in diameter) and maximum follicle diameter each day, as well as the number of follicular waves, were also analyzed for the interovulatory interval studied. Ovulation was detected with ultrasonography as the collapse of a large follicle that had been followed in its growth/static phase for several days. Follicular data were integrated for both ovaries of each ewe.

### 4.3.6 Hormone Analysis

Progesterone (Rawlings et al., 1984), estradiol (Joseph et al., 1992), FSH (Currie and Rawlings, 1989) and LH (Rawlings et al., 1988) concentrations were measured in serum samples by validated RIA procedures. Steroid assays used antisera produced in our laboratory with standards obtained from the Sigma Chemical Company (Joseph et

al., 1992; Rawlings et al., 1984) and the reagents for the gonadotropin assays were obtained from NIDDK/NHPP. The assay sensitivities (defined as the lowest concentration of a hormone capable of significantly displacing radio-labeled hormone from the antibody) were: 0.03 ng/mL for progesterone, 1.0 pg/mL for estradiol and 0.1 ng/mL for FSH and LH. The ranges of standards were: 0.1 to 5 ng/mL, 1.0 to 100 pg/mL, 0.12 to 16.0 ng/mL, and 0.063 to 8.0 ng/mL for the progesterone, estradiol, FSH, and LH assays, respectively. A concentration equivalent to the sensitivity of the assay was assigned to serum samples with hormone concentrations lower than the assay sensitivity. Serum samples collected daily, throughout the experimental period, were analyzed for concentrations of progesterone, estradiol, and FSH. All serum samples collected every 12 min were analyzed for concentrations of LH; FSH was also measured in experiment 1.

For Experiments 1 and 2, the intra- and inter-assay coefficients of variation (CVs) were 11.4% and 9.6% or 7.1% and 13.7% for reference sera with mean progesterone concentrations of 0.26 or 1.17 ng/mL, respectively. The intra- and inter-assay CVs were 9.2% and 11.0% or 6.7% and 11.1% for reference sera with mean estradiol concentrations of 7.83 or 23.36 pg/mL, respectively. The intra- and inter-assay CVs were 4.8% and 7.2% or 7.9% and 11.8% for reference sera with mean LH concentrations of 0.41 or 2.77 ng/mL, respectively. For Experiment 1, The intra-assay CVs were 2.2% or 4.0% for reference sera with mean FSH concentrations of 1.57 or 3.68 ng/mL, respectively. For Experiment 2, the intra-assay CVs were 2.3% or 3.4% for reference sera with mean FSH concentrations of 0.86 or 4.04 ng/mL, respectively.

The PC-PULSAR program (Gitzen and Ramirez, 1988) was used to assess mean and basal serum FSH and LH concentrations as well as FSH and LH pulse frequency and amplitude in blood samples collected every 12 min for 6h. The basal serum level ("smoothed series") was generated after the removal of short-term variation in hormone concentrations, including possible pulses. Standard deviation criteria (G and Baxter parameters) were used for pulse detection.

Peaks of FSH in blood samples taken daily were identified using cycle-detection software (Clifton and Steiner, 1983). A fluctuation or cycle was defined as a progressive rise and fall in hormone concentrations that encapsulated a peak concentration (nadir-to-peak-to-nadir; Clifton and Steiner, 1983). Mean basal FSH concentrations were determined by averaging the lowest points between peaks (nadirs). Follicle stimulating hormone peak concentration was defined as the concentration of FSH observed at the apex of the FSH peak. Follicle stimulating hormone peak amplitude was defined as the difference between the FSH peak concentration and the nadir before the peak concentration.

### 4.3.7 Statistical Analyses

All data for hormone concentrations and ovarian follicles measured daily were normalized to the day of ovulation both for presentation and statistical analyses. Two-way repeated measures ANOVA (Sigma Stat 7 for Windows Version 2.03, 1997, SPSS Inc.; Chicago, IL, USA) was used to assess time trends and treatment effects on hormone concentrations and numbers of follicles in different size classes. Two-way repeated measures ANOVA was also used to assess differences in parameters of LH and FSH secretion in blood samples collected every 12 min for 6 h amongst treated and control ewes and between days of intensive sampling. Two-way repeated measures

ANOVA was used to assess differences in characteristics of FSH peaks (i.e., FSH peak concentration and amplitude, FSH peak duration and basal FSH concentration) and ovarian follicles (i.e., length of growth, static and regression phases, life span and growth rate) amongst peaks/waves detected during the interovulatory interval studied and amongst the treated and control ewes. The t-test was used to compare the number of FSH peaks and follicular waves, interovulatory interval and ovulation rate amongst the treated and control ewes. If the main effects, or their interactions, were significant ($P<0.05$), in the repeated measures ANOVA's, then Fisher's protected least significant difference (LSD) was used as a post-ANOVA test to detect differences between individual means ($P<0.05$). Data are expressed as mean ± S.E.M.

## 4.4 RESULTS

### 4.4.1 Experiment 1

#### 4.4.1.1 Characteristics of Serum LH Concentrations

Ewes were given GnRH every h from 8AM on Day 7 after ovulation to 8AM on Day 11 (96 h). It should be noted that on Day 11 blood sampling started 36 min prior to the last injection of GnRH and continued for 6 h after injection. During the periods of intensive blood sampling, each injection of GnRH was seen to cause an LH pulse. Based on the data from blood samples collected every 12 min for 6 h, treatment with GnRH resulted in an increased LH pulse frequency on Days 7 and 9 compared to control ewes ($P<0.05$; Figure 4.2. C). Mean and basal serum LH concentrations (Figure 4.2. A, B) and LH pulse amplitude (Figure 4.2. D) however, were only greater in GnRH treated ewes and compared to control ewes on the first day of treatment (Day 7 after ovulation; $P<0.05$; Figure 2).

#### 4.4.1.2 Mean Daily Serum Estradiol Concentrations

During the period of treatment with GnRH or saline, serum estradiol concentrations were higher in ewes treated with GnRH compared to control ewes (7.24 ± 0.5 pg/mL vs. 4.59 ± 0.7 pg/mL; $P<0.001$; Figure 4.3A). Comparison of individual means showed that mean serum estradiol concentrations were significantly higher in ewes treated with GnRH compared to control ewes on Days 8 to 11 after ovulation (Figure 4.3A).

#### 4.4.1.3 Mean Daily Serum Progesterone Concentrations

During the period of treatment with GnRH or saline, serum progesterone concentrations were higher in ewes treated with GnRH compared to control ewes (5.35 ± 0.6 ng/mL vs. 3.09 ± 0.3 ng/mL; $P<0.001$; Figure 4.3B). Comparison of individual means showed that mean serum progesterone concentrations were significantly higher in ewes treated with GnRH compared to control ewes on Days 8 to 11 after ovulation (Figure 4.3B).

#### 4.4.1.4 Characteristics of Serum FSH concentrations

Based on blood samples taken every 12 min for 6 h on Day 7, Day 9 and Day 11 after ovulation, FSH secretory profiles were found to be non-pulsatile by the PC-PULSAR program (Gitzen and Ramirez, 1988) and no response to the injection of GnRH was seen ($P>0.05$; Figure 4.4). In ewes treated with GnRH, mean serum FSH concentrations were lower on Days 9 and 11 compared to Day 7 after ovulation; concentrations on Day 9 and 11 were also lower than in control ewes ($P<0.05$; Figure 4.4).

Based on blood samples collected daily, there were no differences in FSH peak concentrations and amplitude, basal FSH concentrations and FSH peak duration amongst control ewes and ewes treated with GnRH ($P>0.05$; Figure 4.5). During the interovulatory interval studied, there were only 4.0 ± 0.0 FSH peaks in GnRH treated ewes compared to 5.0 ± 0.0 in control ewes ($P<0.05$; Figure 4.5). In the interovulatory interval studied, the time from the 2nd FSH peak (Day 4 in both ewes treated with GnRH and control ewes) to the next FSH peak (Day 11 in ewes treated with GnRH and Day 8 in control ewes) was longer in ewes treated with GnRH compared to control ewes (7.43 ± 0.3 vs. 3.83 ± 0.4 d; $P<0.05$; Figure 4.5). The 3rd FSH peak detected on Day 8 in control ewes was missing in ewes treated with GnRH (Figure 4.5).

**4.4.1.5 Antral Follicle Development, ovulations and corpus luteum**

During the interovulatory interval studied, we observed 4 follicular waves with corresponding FSH peaks in control ewes but only 3 follicular waves with corresponding FSH peaks in ewes given GnRH ($P<0.05$; Figure 4.5). The time from the day of emergence of the 2nd follicular wave (Day 4 in both groups) to emergence of the next follicular wave was longer in ewes treated with GnRH compared to control ewes (6.86 ± 0.4 vs. 4.00 ± 0.5 respectively; $P<0.05$; Figure 4.5). The 3rd follicular wave, emerging on Day 8 in control ewes, was missing in ewes treated with GnRH (Figure 4.5). The lifespan of follicles growing in the 2nd follicular wave was longer in ewes treated with GnRH compared to control ewes (14.14 ± 0.3 vs 9.0 ± 0.8 d; $P<0.001$; Figure 4.5). This was due to a prolonged growth phase in ewes given GnRH compared to control ewes (6.43 ± 0.4 vs 3.0 ± 0.3 d; $P<0.001$; Figure 4.5). Interestingly, within GnRH treated ewes, the length of the growth phase of follicles growing in the 2nd

follicular wave was greater than for the first follicular wave (6.43 ± 0.4 vs 3.71 ± 0.4 d; P<0.001; Figure 4.5) but not different from that of the final or ovulatory wave of the interovulatory interval (4.57 ± 0.9 d; P>0.05; Figure 4.5). The mean duration of the interovulatory interval (17.29 ± 0.2 d vs. 17.67 ± 0.2 d; P>0.05; Figure 4.5) and the ovulation rate (2.14 ± 0.3 vs. 1.67 ± 0.2; P>0.05) did not differ among the GnRH treated and control ewes.

During the interovulatory interval studied and as measured daily, there were no differences in the number of small (25.16 ± 1.8 vs 26.57 ± 1.5), medium (2.62 ± 0.7 vs 2.07 ± 0.6) or large sized follicles (2.25 ± 0.4 vs 2.10 ± 0.4) amongst the control ewes and the ewes treated with GnRH (P>0.05). Maximum follicle diameter, measured on a daily basis, was greater in ewes given GnRH compared to control ewes during the period of treatment with GnRH (6.43 ± 0.3 mm vs. 5.56 ± 0.3 mm; P<0.001; Figure 4.6). Comparison of individual means showed that maximum follicle diameter measured daily was significantly higher in ewes treated with GnRH compared to control ewes from 10 to 15 d after ovulation (P<0.05; Figure 4.6). Maximum diameter of the corpus luteum, measured on a daily basis, did not differ between the control ewes and ewes treated with GnRH during the treatment period (10.73 ± 0.4 vs 10.86 ± 0.8 mm; P>0.05).

### 4.4.2 Experiment 2:

#### 4.4.2.1 Mean Daily Serum Progesterone Concentrations

Serum progesterone concentrations were higher in ewes treated with implants releasing progesterone compared to control ewes (5.28 ± 0.8 ng/mL vs. 2.55 ± 0.4 ng/mL; P<0.001; Figure 4.7) during the period of treatment with progesterone releasing

implants. Comparison of individual means showed that mean serum progesterone concentrations were significantly higher in ewes treated with implants compared to control ewes on Days 5 to 13 after ovulation ($P<0.05$; Figure 4.7).

#### 4.4.2.2 Characteristics of Serum LH Concentrations

Ewes were given progesterone releasing implants on Day 4 after ovulation, implants were removed on Day 14. Based on data from blood samples collected every 12 min for 6 hrs, treatment with progesterone releasing implants decreased LH pulse frequency on day 10 compared to day 3 after ovulation and on Day 10, pulse frequency was lower than in control ewes, ($P<0.05$; Figure 4.8). Basal serum LH concentrations were increased on Day 16 after ovulation in both groups of ewes but concentrations in control ewes on day 16 exceeded those treated with progesterone implants ($P<0.05$; Figure 4.8).

#### 4.4.2.3 Characteristics of Serum FSH concentrations

Based on blood samples collected daily, there were no differences in the number of FSH peaks, FSH peak concentration, amplitude and duration, or basal FSH concentration amongst control ewes and ewes treated with progesterone releasing implants ($P>0.05$; Figure 4.9).

#### 4.4.2.4 Antral Follicle Development and Ovulations

The number of follicle waves emerging per animal did not differ amongst implant treated and control ewes ($P>0.05$; Figure 4.9) during the interovulatory interval studied. All implant treated and control ewes had four follicular waves with corresponding FSH peaks. There were no differences in growth characteristics (growth, static, and regressing phase, growth rate and lifespan of the largest follicle in a wave) of

follicular waves amongst implant treated ewes and control ewes (P>0.05; Figure 4.9). The mean duration of the interovulatory interval (19.33 ± 0.6 d vs. 18.0 ± 0.4 d; P>0.05; Figure 4.9) and the ovulation rate (2.33 ± 0.3 vs. 1.83 ± 0.2; P>0.05) did not differ among the implant treated and control ewes. During the interovulatory interval and as measured daily, there were no differences in the number of small (22.77 ± 1.3 vs 21.95 ± 1.5), medium (1.46 ± 0.4 vs 1.58 ± 0.5) or large follicles (1.70 ± 0.3 vs 1.68 ± 0.4) or the daily maximum follicle diameter (5.61 ± 0.4 vs 5.17 ± 0.4 mm) amongst control ewes and implant treated ewes (P>0.05).

#### 4.4.2.5 Mean Daily Serum Estradiol Concentrations

Mean serum estradiol concentrations did not differ amongst progesterone treated and control ewes during the period of treatment with progesterone releasing implants (5.50 ± 1.0 pg/mL vs 4.72 ± 1.4 pg/mL; P>0.05).

Fig. 4.1. Schematic representation of the experimental designs used in the present study. In experiment 1, injections of GnRH (200ng; IV) were given every h for 96 h starting on Day 7 after ovulation (Day 0). In experiment 2, progesterone implants were inserted subcutaneously 4 d after ovulation and remained in place for 10 d. Control animals received saline or empty implants in experiment 1 and 2, respectively. Daily transrectal ovarian ultrasonography and blood sampling was performed on all ewes starting from the day when the ewe was marked with a vasectomized crayon-harnessed ram (estrus) until ovulation at the end of cycle. Blood samples were also taken every 12 min for 6h (intensive bleeds) on Day 7, Day 9 and Day 11 after ovulation in experiment 1 and on Day 3, Day 10 and Day 16 in experiment 2, to characterize the pulsed secretion of LH and or FSH.

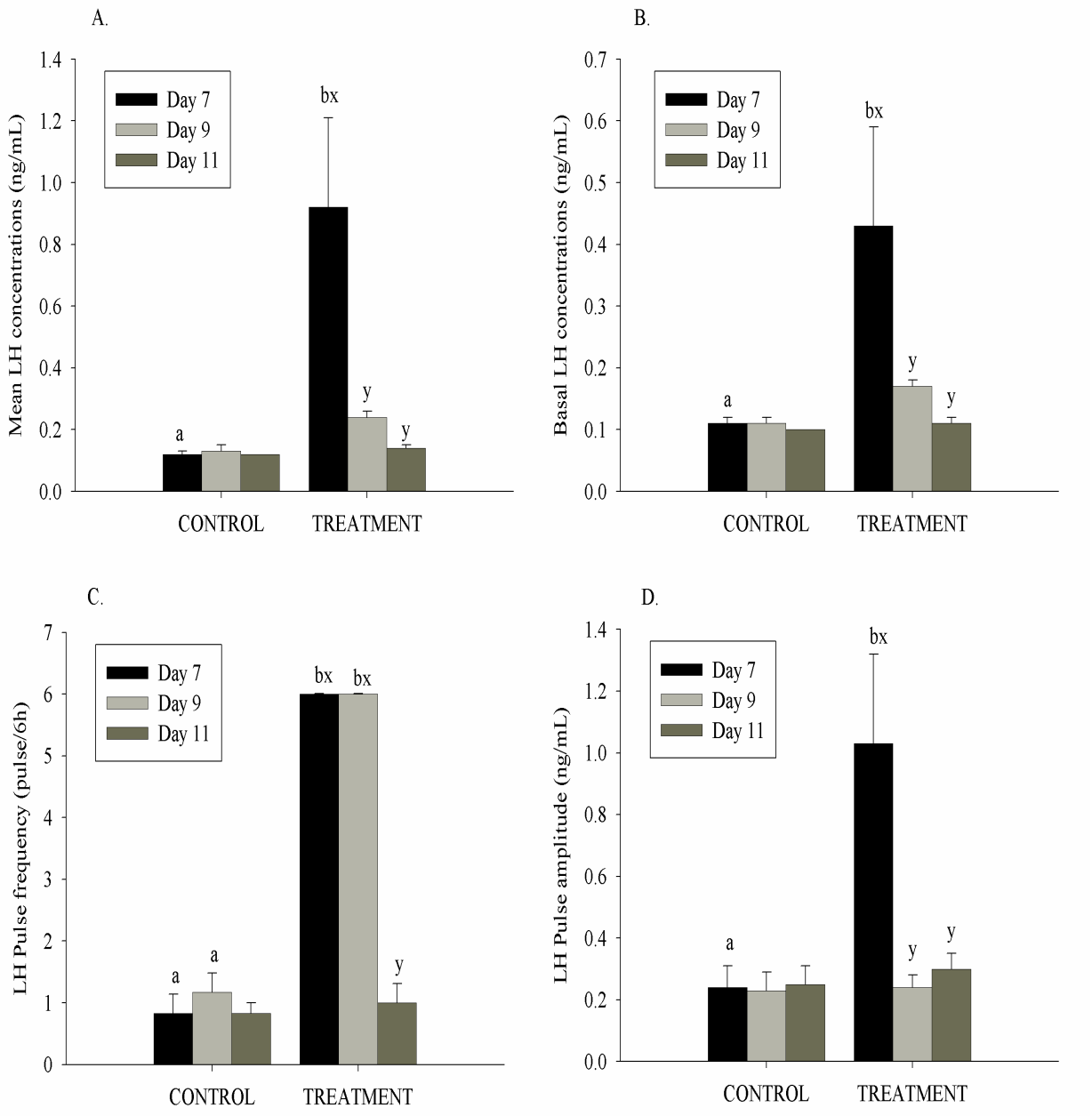


Fig. 4.2. The characteristics of pulsatile LH secretion (mean and basal serum LH concentrations and LH pulse frequency and amplitude; mean ± S.E.M) determined from serum samples collected every 12 min for 6 h on Day 7 (black bars), Day 9 (light gray bars) and Day 11 (dark grey bars) after ovulation in cyclic Western White Face ewes. Starting on Day 7 after ovulation, ewes were treated with GnRH (200ng; IV; treatment group) or saline (control group) every h for 96 h. Letters (a-b) indicate differences between control ewes and ewes treated with GnRH ($P<0.001$) within the respective intensive sampling period. Letters (x-y) indicate differences between the days of intensive blood sampling ($P<0.001$) for control ewes or ewes treated with GnRH. [n=6 (control), n=7 (treatment)].

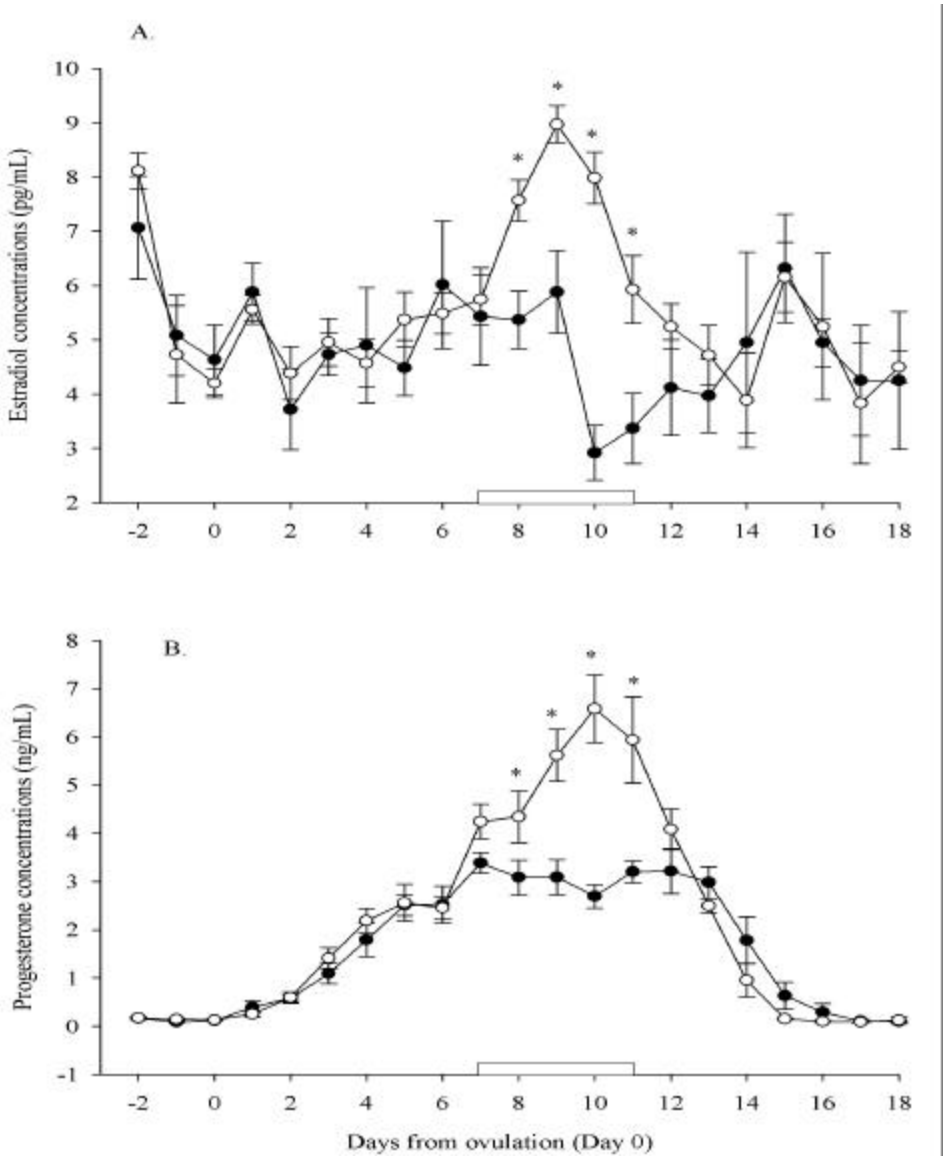


Fig. 4.3. Mean (±S.E.M.) daily serum estradiol (Panel A) and progesterone concentrations (Panel B) over the entire experimental period in cyclic Western White Face ewes treated every h for 96 h, with GnRH (200ng; IV; open circles; n=7) or saline (black circles; n=6), starting on Day 7 after ovulation (Day 7-11; indicated by an open rectangular box on the X-axis). Data were normalized to the day of ovulation (Day 0) in all ewes. Asterisks (*) indicate differences between ewes treated with GnRH and control ewes ($P<0.001$).

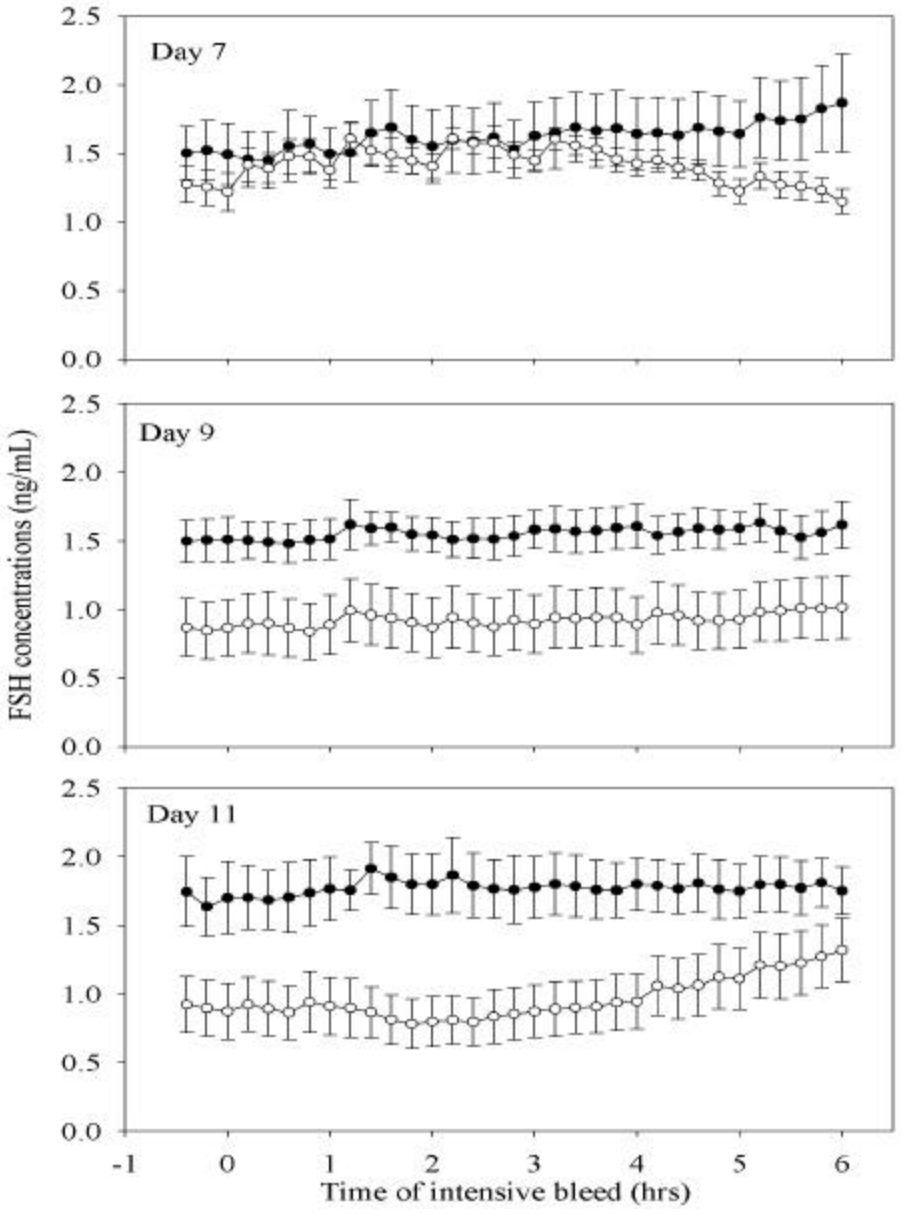


Fig. 4.4. Mean (±S.E.M.) serum FSH concentrations determined from serum samples collected every 12 min for 6 h on Day 7 (top panel), Day 9 (middle panel) and Day 11 (bottom panel) after ovulation in cyclic Western White Face ewes treated every h for 96 h, with GnRH (200ng; IV; open circles; n=7) or saline (black circles; n=6), starting on Day 7 after ovulation.

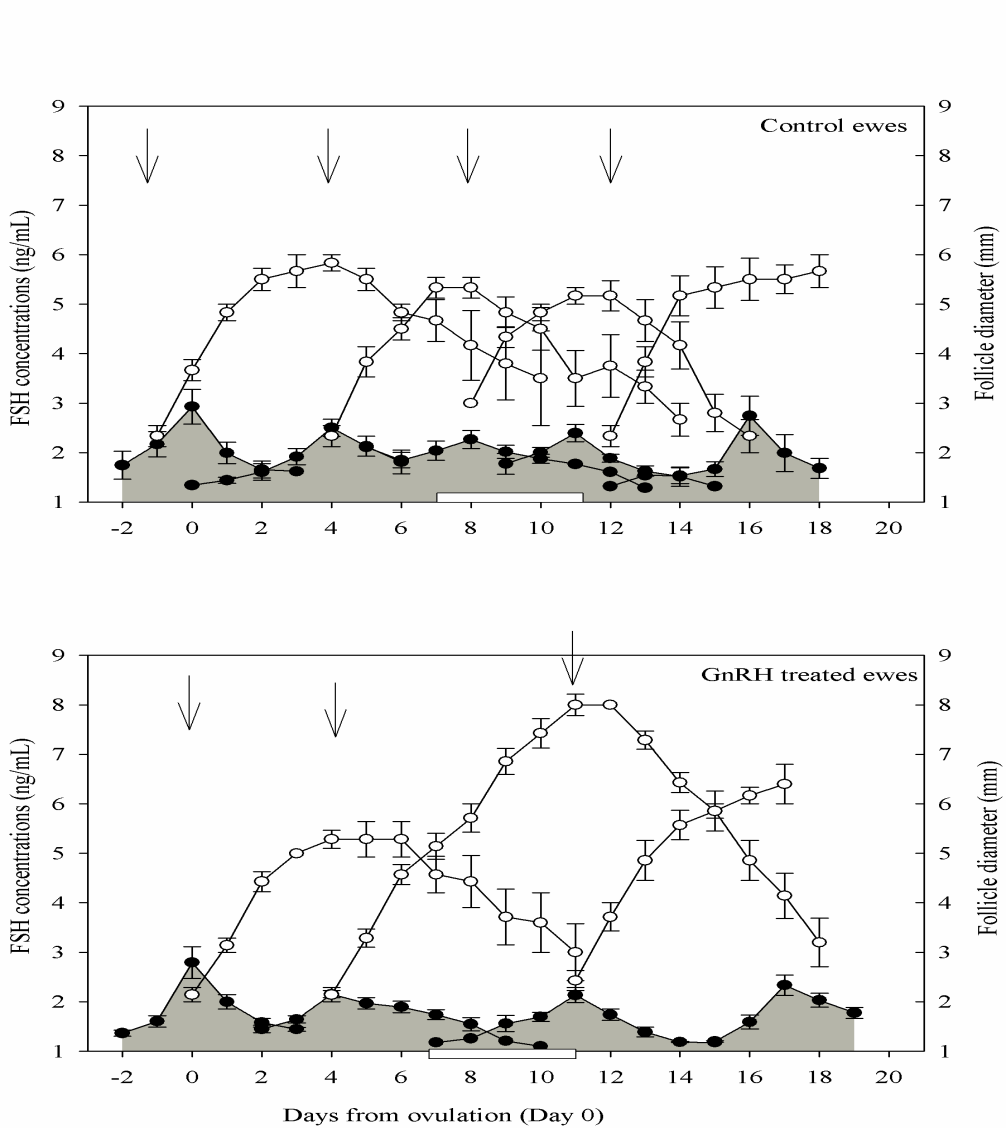


Fig. 4.5. Peaks in serum concentrations of FSH (outlined with shading) and their associated emerging follicular waves (open circles) in cyclic Western White Face ewes treated every  h for 96 h, with GnRH (200ng; IV; bottom panel; n=7) or saline (top panel; n=6), starting on Day 7 after ovulation (Day 7-11; indicated by an open rectangular box on the X-axis). Data were normalized to the day of ovulation (Day 0) in all ewes. Concentrations of FSH and follicle diameters are expressed as mean ± S.E.M. The average curves representing the growth, static and regression phases of all ewes in a group were normalized for each follicle wave to the mean day of wave emergence (indicated by the arrows; Barrett et al., 2006). All FSH peaks for all ewes are shown normalized to the mean day of occurrence of the apex of the FSH peak for each wave. Although most follicular waves emerge at the zenith of the FSH peak, emergence can occur one day before or after the zenith of the FSH peak. For every FSH peak, serum concentration profiles were delimited by the encompassing nadirs of the FSH concentrations (hence the overlap of the data for adjacent peaks in some cases).

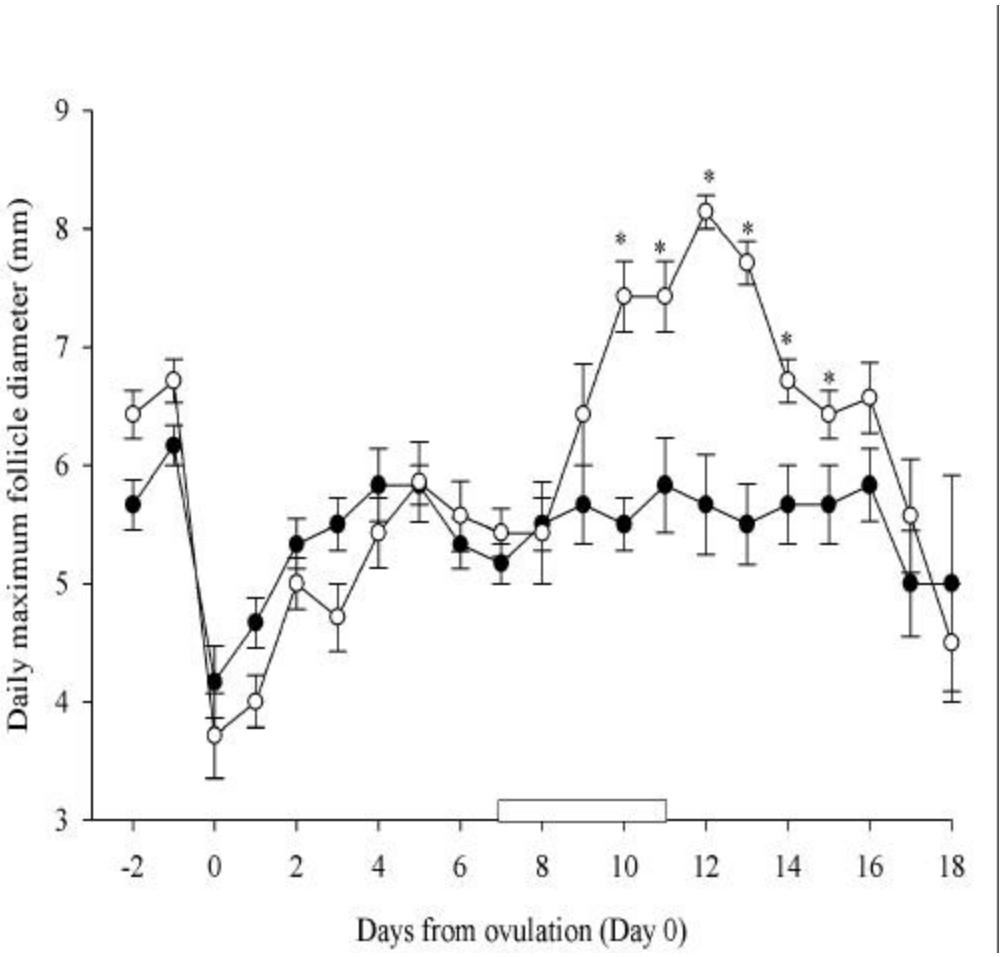


Fig. 4. 6. Mean (±S.E.M.) daily maximum follicle diameter over the entire experimental period in cyclic Western White Face ewes treated every h for 96 h, with GnRH (200ng; IV; open circles; n=7) or saline (black circles; n=6), starting on Day 7 after ovulation (Day 7-11; indicated by an open rectangular box on the X-axis). Data were normalized to the day of ovulation (Day 0) in all ewes. Asterisks (*) indicate differences amongst ewes treated with GnRH and control ewes ($P<0.001$).

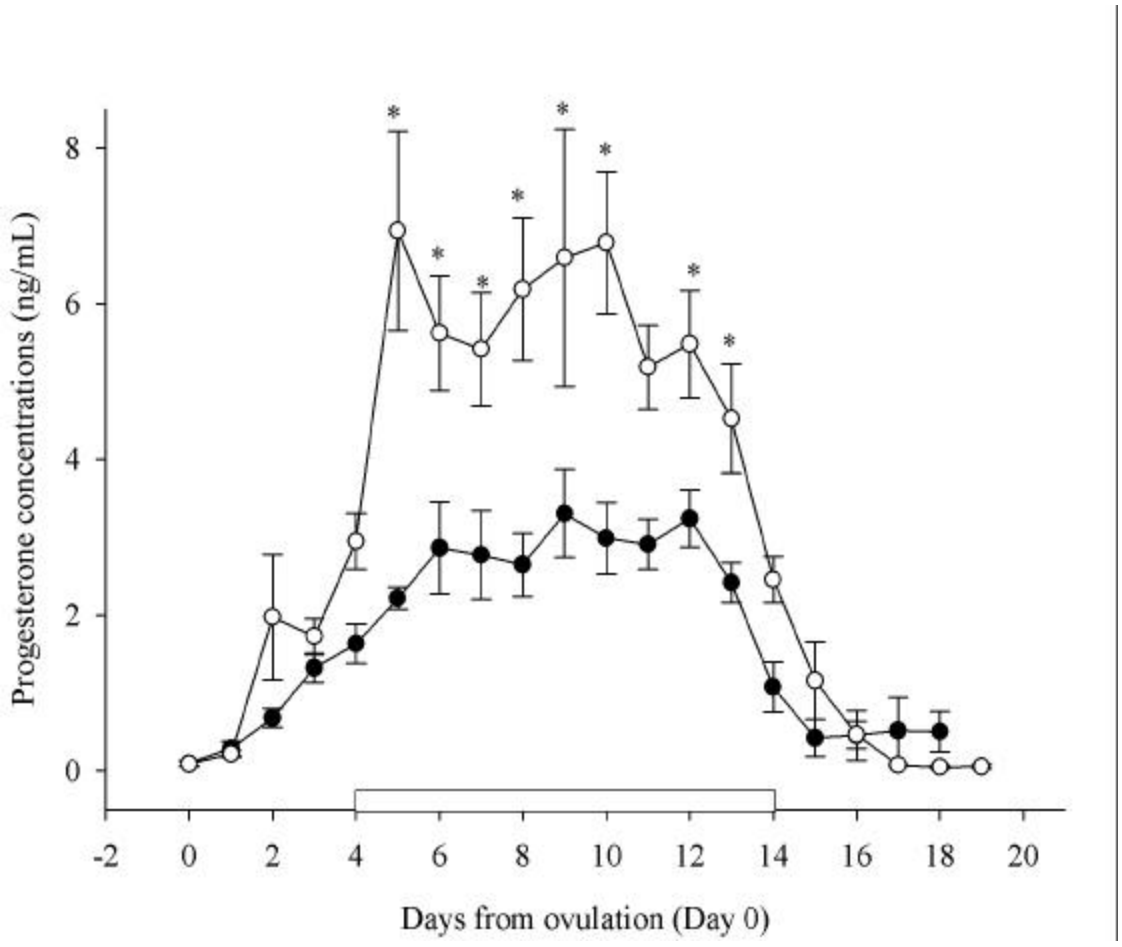


Fig. 4 7. Mean (±S.E.M.) daily serum progesterone concentrations over the entire experimental period in cyclic Western White Face ewes treated for 10 d, from Day 4 after ovulation (Day 4-14; indicated by an open rectangular box on the X-axis) with silastic rubber implants (s.c) containing 10% progesterone (open circles; n=6) and sham operated control ewes (black circles; n=6). Data were normalized to the day of ovulation (Day 0) in all ewes. Asterisks (*) indicate differences between ewes treated with GnRH and control ewes ($P<0.001$).

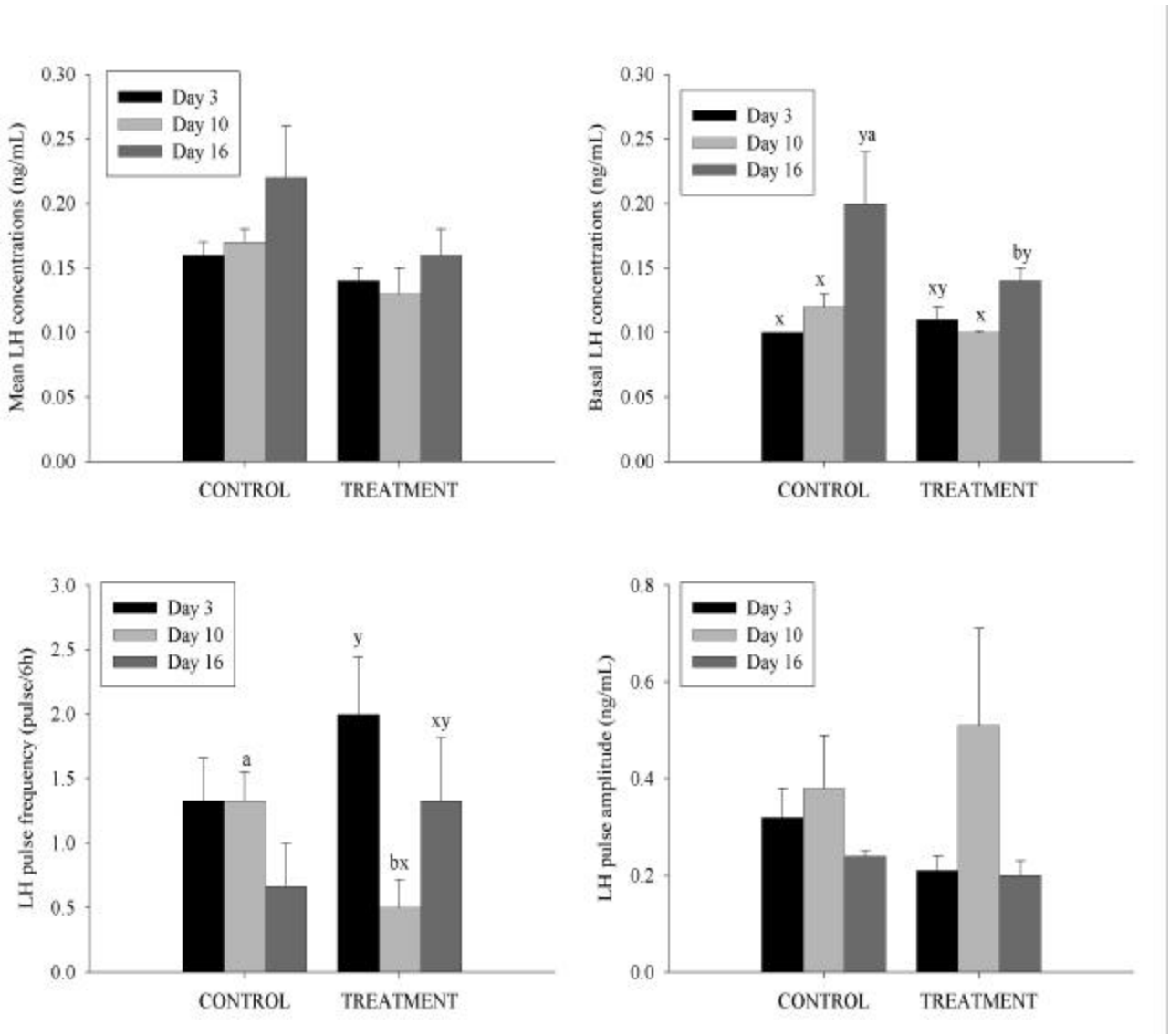


Fig. 4.8. The characteristics of pulsatile LH secretion (mean and basal serum LH concentrations and LH pulse amplitude and frequency; mean ± S.E.M) determined from serum samples collected every 12 min for 6 h on Day 3 (black bars), Day 10 (light gray bars) and Day 16 (dark grey bars) after ovulation in cyclic Western White Face ewes. Ewes were treated for 10 d, from Day 4 after ovulation with silastic rubber implants (s.c) containing 10% progesterone (n=6) and sham operated control ewes (n=6). Letters (a-b) indicate differences between control ewes and ewes treated with progesterone releasing implants ($P<0.001$) within respective intensive sampling period. Letters (x-y) indicate differences between the intensive blood sampling days ($P<0.001$) for control ewes or ewes treated with progesterone releasing implants. [n=6 (control), n=6 (treatment)].

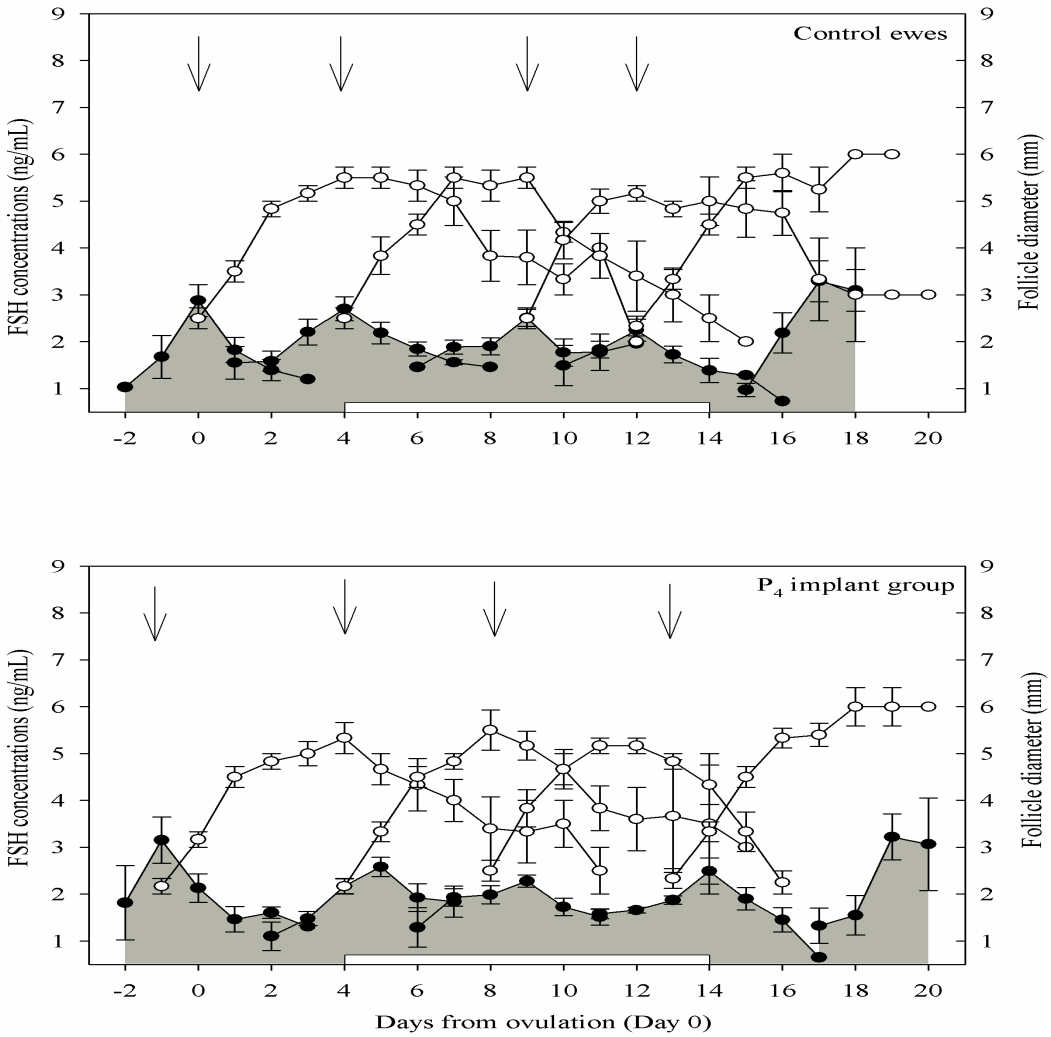


Fig. 4.9. Peaks in serum concentrations of FSH (outlined with shading) and their associated emerging follicle waves in cyclic Western White Face ewes treated for 10 d, from Day 4 after ovulation (Day 4-14; indicated by an open rectangular box on the X-axis) with silastic rubber implants (s.c) containing 10% progesterone (bottom panel; n=6) and sham operated control ewes (top panel; n=6). Data were normalized to the day of ovulation (Day 0) in all ewes. Concentrations of FSH and follicle diameters are expressed as mean ± S.E.M. The average curves representing the growth, static and regression phases of all ewes in a group were normalized for each follicle wave to the mean day of wave emergence (indicated by the arrows; [38]). All FSH peaks for all ewes are shown normalized to the mean day of occurrence of the apex of the FSH peak for each wave. Although most follicular waves emerge at the zenith of the FSH peak, emergence can occur one day before or after the zenith of the FSH peak. For every FSH peak, serum concentration profiles were delimited by the encompassing nadirs of the FSH concentrations (hence the overlap of the data for adjacent peaks in some cases).

## 4.5 DISCUSSION

In Experiment 1 of the present study, GnRH was given to ewes in the luteal phase of the cycle when LH pulse frequency was very minimal. The treatment created LH pulses with a frequency similar to the follicular phase of an estrous cycle (Baird, 1978; Karsch et al., 1979). The amplitude of the induced pulses on the first day of treatment (Day 7 after ovulation) was about four-fold greater than that seen in control ewes but amplitude decreased to control values by Day 9 after ovulation and remained at that range until the end of the treatment (Day 11). Based on serum samples taken every 12 min, FSH secretion was non-pulsatile and showed no response to GnRH. This is not unexpected as FSH secretion is largely constitutive and does not occur in response to GnRH pulses (McNeilly et al., 2003; Wallace and McNeilly, 1986; Wheaton et al., 1984). It is interesting that creation of an LH pulse frequency, analogous to the follicular phase, in the present ewes, during the luteal phase of their cycle, increased follicular growth and estradiol secretion compared to control ewes. The effect on maximum follicle size was not significant until Day 10 after ovulation or after 3 days of GnRH treatment. The initial increase in LH pulse amplitude in response to GnRH had disappeared by Day 10 after ovulation. A significant increase in serum concentration of estradiol did occur by Day 8 but was sustained until Day 11. LH has been shown to stimulate estradiol production by way of receptors on the theca cells and later, as follicles mature, directly on the granulosa cells themselves (Baird, 1978; Hanukoglu., 1992; Martin, 1984). These effects on antral follicle growth and function, even in a mileu of high concentrations of progesterone, in experiment 1 of the present

study, would appear to have occurred largely due to the increase in LH pulse frequency. This contention is reinforced by the fact that serum concentrations of FSH declined over the period of GnRH treatment. The significant increase in basal serum concentrations of LH on Day 16 after ovulation in experiment 2 of the present study may reflect luteolysis and removal of progesterone negative feedback on basal LH secretion (Rawlings and Cook, 1993).

The large antral follicles of Wave 2 of the cycle, that were exposed to the GnRH-induced increase in LH pulse frequency, grew to a larger size than the equivalent follicles in the control ewes or even the follicles in Wave 1 of GnRH-treated ewes. The follicles of Wave 2 in GnRH-treated ewes grew and functioned in terms of estradiol production, like ovulatory follicles growing at the end of a cycle even in a mileu of high serum concentrations of progesterone. The follicles of Wave 2 in GnRH-treated ewes were not persistent but had a similar growth phase to ovulatory follicles in the control ewes but then regressed. It is intriguing that a follicular wave reminiscent of an ovulatory wave can be induced in the luteal phase of the cycle by increasing LH pulse frequency to that seen during the follicular phase. Clearly the presence of the serum concentrations of progesterone of the luteal phase do not directly inhibit a follicle from growing to ovulatory sizes when exposed to a significant increase in LH pulse frequency. However, the presence of progesterone did prevent the endocrine cascade that precedes and causes ovulation in the follicular phase of a cycle (Karsch et al., 1979). Preovulatory follicles at the end of the cycle in ewes tend to be somewhat larger and more estrogenic than the largest follicles seen in other follicular waves in the cycle (Ravindra et al., 1994). However, the differences are small and rather variable. In the

normal follicular phase in the ewe, LH pulse frequency is enhanced, compared to the luteal phase, but amplitude is actually reduced (Rawlings and Cook, 1993). This again emphasizes the role of increased LH pulse frequency in the final growth and development of ovulatory follicles in the ewe.

In Experiment 1, FSH secretion was suppressed during the period of GnRH treatment, compared to control ewes. In contrast to control ewes, there was no FSH peak at Day 8 after ovulation, on the second day of treatment, in GnRH-treated ewes. This resulted in the loss of a follicular wave. We assume that the increased serum concentrations of estradiol and progesterone caused by GnRH treatment suppressed FSH secretion and blocked the peak in serum concentrations of FSH. Inhibin is also important in the negative feedback regulation of FSH secretion in the ewe but inhibin was not measured in the present study (Campbell et al., 1995; Scaramuzzi et al., 1993). The existence of follicular dominance in the ewe, or the ability of secretory products of a large follicle to suppress the emergence and growth of other follicles, has been debated in the ewe (Evans, 2003b). Some form of limited dominance has been suggested (Evans, 2003b). In previous studies using subcutaneous implants releasing estradiol and progesterone, we have shown that supraphysiological serum concentrations of estradiol and progesterone suppressed FSH peaks (Barrett et al., 2006; Barrett et al., 2007; Rawlings et al., 1984). We regard the doubling of serum concentrations of estradiol and progesterone, seen in GnRH treated ewes in the present study, as supraphysiological (Barrett et al., 2006; Barrett et al., 2007).

In those previous studies, when the amplitude of FSH peaks were decreased, follicular waves ceased but discernable FSH peaks continued to occur at the expected

frequency (Barrett et al., 2006; Barrett et al., 2007); such peaks appear to continue in ovariectomized ewes (Duggavathi et al., 2005a). When we have created FSH peaks in cyclic ewes during the interwave interval by injections of oFSH, follicular waves were induced even if the oFSH was given during the growth phase of a follicular wave resulting from an endogenously generated FSH peak (Duggavathi et al., 2004). In addition, FSH peaks created by injection of oFSH did not disrupt the normal rhythm of waves (Duggavathi et al., 2004). Injections of oFSH created physiological peaks in serum concentrations of FSH (Barrett et al., 2006; Duggavathi et al., 2004). The studies above pose questions regarding the existence of antral follicular dominance in the ewe and suggest that the rhythm of FSH peaks in the ewe are not governed by changes in feedback regulation by secretory products of the large antral follicles in follicular waves but perhaps some yet unknown endogenous rhythm. It is intriguing that following the missed FSH peak and follicular wave, in GnRH-treated ewes in the present study, the next FSH peak and emergence of a wave occurred on Day 11 at the same time as in control ewes. In other words, in GnRH-treated ewes, an FSH peak was skipped but this did not perturb the overall rhythm of FSH peaks and follicular waves. However, it did appear that GnRH (LH) treatment enhanced the growth and estradiol production of the largest follicle of Wave 2 of the interovulatory interval suppressing the next expected FSH peak and follicular wave. Based on follicle growth and serum concentrations of estradiol, it could be argued that this induced dominance appeared to wane prior to the FSH peak and follicular wave on Day 11.

In Experiment 1 of the present study, serum concentrations of progesterone doubled in response to GnRH treatment. Progesterone secretion in the ewe is pulsatile

and pulses may or may not be driven by a pulse of LH secretion (Hanukoglu, 1992; McNeilly et al., 1992). Clearly enhancing LH pulse frequency from the minimal values of the luteal phase to values similar to the follicular phase enhanced the ability of the corpus luteum to secrete progesterone.

In Experiment 2 of the present study, ewes were given progesterone-releasing implants during the luteal phase of an estrous cycle. The resulting serum progesterone concentrations were twice those seen in control ewes. Progesterone treatment decreased the frequency of secretion of LH pulses to less than half that seen in control ewes. The frequency of LH pulses is at a minimum during the luteal phase of the cycle in normal ewes and at a maximum of = 1 pulse per h during the follicular phase (Goodman et al., 1981a; Karsch et al., 1979; Karsch et al., 1980; Rawlings and Cook, 1993). The combined negative feedback effects of progesterone and estradiol are known to powerfully suppress LH pulse frequency (Goodman et al., 1981a; Karsch et al., 1979; Karsch et al., 1980; Rawlings et al., 1984). The significant suppression of LH pulse frequency by progesterone, in the present study, to below that expected in a normal luteal phase, did not have any effect on the emergence, growth and regression of follicles growing in follicular waves. Serum concentrations of FSH were not significantly affected. In the cyclic ewe, the negative feedback effects of progesterone on FSH secretion are not as marked or as consistent as for LH (Roche and Ireland, 1981).

In the cyclic ewe, follicular waves emerge every 3 to 5 d (Bartlewski et al., 1999a; Schrick et al., 1993; Souza et al., 1998). Each wave is heralded by a peak in serum concentrations of FSH and these peaks are essential triggers for the emergence of

each wave (Bartlewski et al., 1999a; Duggavathi et al., 2003; Evans et al., 2000; Ginther et al., 1995). However, when the secretory patterns of LH were correlated with the characteristics of follicular waves, both during the period from metestrus to the early luteal phase or during the mid to late luteal period of the estrous cycle, no consistent and major regulatory interactions were indicated (Bartlewski et al., 2000a; Duggavathi et al., 2005b). Ewes in these two studies would have experienced frequencies of pulsed LH secretion ranging from the low frequencies of the mid to late luteal phase to the higher frequencies of the early luteal phase of the cycle. The characteristics of follicular waves studied included interwave interval, numbers of follicles in a wave, maximum follicle diameter, follicle lifespan subdivided into its phase, as well as the growth and regression rates of follicles (Bartlewski et al., 2000a; Duggavathi et al., 2005b).

In a recent study (Campbell et al., 2007), using ewes with ovarian transplants and given a GnRH antagonist, antral follicles grew from 4.5 mm to 4.9 mm in diameter, over a period of 66 h with only basal LH secretion. This change in size was not significant and was not enhanced by treatment with pulses of LH or constant infusion of a low dose of LH over a similar time period. However, increasing the dose of LH given by constant infusion gave a significant increase in follicle size (3.9 to 5.0 mm in diameter) (Campbell et al., 2007). These follicles luteinized but did not ovulate in response to hCG. Suppression of the frequency of the pulsed secretion of LH in the present study, in intact cyclic ewes, to less than half of the frequency of the luteal phase of the estrous cycle, was consistent with normal follicular wave emergence and growth. These findings indicate that in the cyclic ewe only very low serum concentrations of LH, with very few pulses, are required to support ovarian follicular wave emergence and growth.

In conclusion, decreasing LH pulse frequency significantly, to values lower than the minimal frequencies seen in ewes in the luteal phase of an estrous cycle, did not affect any aspect of the growth and regression of follicular waves. Creating an LH pulse frequency, typical of the follicular phase, in ewes in the luteal phase of a cycle enhanced follicle growth and serum concentrations of estradiol and progesterone. Follicles growing in the luteal phase of a cycle in the ewe, in the presence of progesterone, can grow and function like ovulatory follicles growing in the follicular phase of the cycle, if exposed to an LH pulse frequency similar to the follicular phase. In GnRH-treated ewes, enhanced secretion of estradiol and progesterone suppressed FSH peaks, blocking the emergence of a follicular wave. Loss of a follicular wave did not affect the timing of the next follicular wave when compared to control ewes.

# Chapter 5: EFFECTS OF TREATMENT WITH ESTRADIOL AND PROGESTERONE ON LH AND FSH SECRETION, WAVES OF OVARIAN ANTRAL FOLLICLE GROWTH AND THE SMALL FOLLICLE POOL FROM WHICH FOLLICULAR WAVES EMERGE IN ANESTROUS EWES [*]

Seekallu SV, Toosi BM, Ziegler AC and Rawlings NC

## 5.1 ABSTRACT

In the ewe, ovarian antral follicles emerge or grow in a wave-like pattern and each wave is preceded by a peak in serum FSH concentrations. The purpose of the current study was to see whether in anestrous ewes, the combination of progesterone and estradiol affects FSH peak secretion and numbers of small follicles as was seen with a similar treatment in cyclic ewes. Five ewes received subcutaneous silastic rubber implants (10 x 0.47 cm) containing 10% estradiol-17ß w/w and the other five ewes received the same estradiol implant along with subcutaneous silastic rubber implants (11x 0.48 cm) containing 10% progesterone w/w for 12 d. Daily transrectal ovarian ultrasonography and blood sampling was performed from 5 d before to 9 d after the period the implants were in place. Blood samples were also taken every 12 min for 6 h on Day -2, 6 and 13 prior to or after implant insertion (Day 0 is the day of implant insertion). Luteinizing hormone pulsatility was abolished by the implants ($P<0.05$). During the implant treatment period, FSH peak amplitude was lower in ewes treated with implants releasing estradiol and progesterone compared to ewes treated with implants releasing only estradiol ($P<0.05$). No follicular waves emerged during implant treatment in both groups of ewes ($P<0.05$). The number of FSH peaks did not differ compared to before implant treatment ($P>0.05$). For the ewes treated with estradiol- and progesterone-releasing implants, the number of small follicles was greater during and

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after implant treatment compared to the period before implant treatment ($P<0.05$). To conclude, supra-physiological concentrations of estradiol completely abolished LH pulsatility and blocked follicular wave emergence; the FSH secretory peaks that precede follicular waves were not affected. Supra-physiological concentrations of estradiol-17ß with physiological concentrations of progesterone decreased FSH peak amplitude, abolished LH pulses but did not decrease the size of the small follicle pool in anestrous ewes.

## 5.2 INTRODUCTION

It appears that growth of ovarian antral follicles in the ewe beyond 2 to 3 mm in diameter is gonadotrophin dependent but the dependency of follicles in this size range and below is unclear (Campbell et al., 1995; Scaramuzzi et al., 1993). In the ewe, one to three antral follicles emerge or grow from a pool of small follicles (2-3 mm in diameter) every four to five days (Bartlewski et al., 1999a; Bartlewski et al., 1998; Schrick et al., 1993; Souza et al., 1996). This production of waves of antral follicles occurs in cyclic (Bartlewski et al., 1999a; Schrick et al., 1993) and anestrous ewes (Bartlewski et al., 1998; Souza et al., 1996) and waves end in regression or ovulation. Waves are preceded by a peak in serum concentrations of FSH lasting several days; this peak initiates the wave. The pattern of pulsatile LH secretion during the estrous cycle or during anestrous does not appear to be related to the rhythm of follicular waves in any way (Bartlewski et al., 1999a; Bartlewski et al., 2000a; Bartlewski et al., 2000c; Duggavathi et al., 2004; Duggavathi et al., 2005b; Ginther et al., 1995).

During the breeding season, estradiol and progesterone exert a combined negative feedback effect to maintain the pulsatile secretion of LH at a physiological

frequency and amplitude; the regulation of FSH secretion by steroids is less clear (Goodman et al., 1981a; Goodman et al., 1981b; Karsch et al., 1980; Rawlings et al., 1984). Treatment of cyclic ewes with implants releasing estradiol and progesterone truncated the FSH peaks that precede follicular waves, and blocked wave emergence but did not affect pulsed LH secretion (Barrett et al., 2007). The pool of small ovarian follicles (2-3 mm diameter) from which follicular waves emerge was reduced in number indicating a dependence on the rhythmic production of peaks in serum concentrations of FSH (Barrett et al., 2007). Although follicular waves and their associated peaks in serum FSH concentrations continue during anestrous, circulating concentrations of FSH, LH and estradiol are lower than during the breeding season (Evans et al, 2001a; McNatty et al., 1984b; Walton et al., 1980; Yuthasastrakosol et al., 1977). Estradiol exerts a more powerful negative feedback effect on LH secretion during anestrous than the breeding season (Goodman et al., 1981a; Joseph et al., 1992; Martin et al., 1983).

In chapter 2 of this thesis an experiment was discussed in which treatment of anestrous ewes with, estradiol-releasing implants completely abolished LH pulsatility, blocking follicular wave emergence. Interestingly, in these ewes, FSH secretion was only minimally affected and the pool of small ovarian follicles was not changed. Based on this study, we concluded that although peaks in FSH secretion initiate follicular waves in the anestrous ewe, this also requires some level of pulsed LH secretion. Data presented in chapter 2, also showed that when pulsed secretion of LH was restored by GnRH injections in anestrous ewes, given the estradiol-releasing implants, follicular wave emergence resumed. In the present study, we treated anestrous ewes with implants releasing progesterone and estradiol to see if the combined treatment would reduce the

amplitude of FSH peaks and in turn reduce the numbers of small follicles in the ovary as was seen with the similar treatment in the breeding season. We hypothesized that estradiol and progesterone in combination would suppress the secretory peaks of FSH secretion and reduce the numbers of small antral follicles in the ovary in the anestrous ewe.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Animals

Care and handling of experimental animals was done according to the Canadian Council on Animal Care's published guidelines. Sexually mature, clinically healthy, anestrous (June), WWF ewes (mean body weight of 81.0 ± 4.0 kgs) were kept outdoors in sheltered paddocks. Ewes were fed with a maintenance diet of hay; cobalt-iodized saltlicks and water were freely available. The WWF is a cross between the Columbia and Rambouillet breeds.

### 5.3.2 Ultrasound Technique

Ovarian antral follicular dynamics were monitored in all ewes by transrectal ovarian ultrasonography (scanning) using a 7.5-MHz transducer stiffened with a hollow plastic rod and connected to a B-mode, real-time echo camera (Aloka SSD-900, Overseas Monitor, Richmond, BC, Canada). This technique has been validated for monitoring ovarian follicular dynamics and for CL detection in sheep (Bartlewski et al., 1998; Duggavathi et al., 2003). All images were viewed at a magnification of X 1.5 with constant gain and focal point settings. Ovarian images were recorded (Panasonic AG 1978, Matsushita Electric, Mississauga, ON, Canada) on high-grade video tapes (Fuji S-VHS, ST-120 N, Fujifilm, Tokyo, Japan) for later examination. The relative

position and dimension of follicles and luteal structures were also sketched on ovarian charts.

### 5.3.3 Blood Sampling

Blood samples (10 mL) taken daily were collected by jugular venipuncture into Vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA). Intensive blood sampling every 12 min for 6 h was done via indwelling jugular catheters (5 ml/sample; vinyl tubing, 1.0 mm inside diameter x 1.5 mm outside diameter; SV70, Critchley Electrical Products Pty Ltd., Auburn, NSW, Australia).

All samples were permitted to clot at room temperature for 18 to 24 h. Samples were then centrifuged for 10 min at 1500 x g, and serum was removed and kept at !20 $^{o}$C until assayed.

### 5.3.4 Experimental Design

Five ewes (control group) received subcutaneous silastic rubber implants (10 x 0.47 cm) containing 10% estradiol-17ß w/w (95 mg/implant; Sigma Chemical Company, St. Louis, MO, USA (Barrett et al., 2006)) and the other five ewes received the same estradiol implant along with subcutaneous silastic rubber implants (11x 0.48 cm) containing 10% progesterone w/w (125 mg/implant; Sigma-Aldrich, Oakville, ON, Canada; Barrett et al., 2007) for 12 days. To make the implants, liquid silastic rubber (A-101 medical grade silicone elastomer; Factor II, Inc., Lakeside, AZ, USA) was mixed with the steroid and a curing catalyst was added (Catalyst; Factor II, Inc., Lakeside, AZ, USA). The estradiol mixture was injected into silastic tubing (Silastic laboratory grade tubing; 0.34 cm i.d. x 0.47 cm o.d.; Dow Corning, Midland, MI, USA) and, once cured, the tubing was cut into 10 cm lengths. The progesterone mixture was

injected into Tygon tubing moulds (0.48 cm i.d.; Norton Plastics, Akron, OH). Once cured, the progesterone implants were removed from the moulds, and tubing was cut into 11cm lengths. Implants were soaked in sterile 0.9% (w/v) saline for 36-48 h at room temperature before insertion. Lidocaine hydrochloride (2%; Xylocaine; AstraZeneca Canada Inc., Mississauga, ON, CA) was used as a local anesthetic. A 1.5 cm incision was made in the axillary region with a scalpel, the implant was inserted using a trocar, and the incision was closed with wound clips (9 mm MikRon AUTOCLIP; Becton Dickinson Primary Care Diagnostics, Sparks, MD, USA). All implants were removed from all ewes 12 d after insertion (Day 0= day of insertion). Daily transrectal ovarian ultrasonography was performed from 5 d before to 9 days after the period the implants were in place. Daily blood samples were collected for the entire trial period. Blood samples were also taken every 12 min for 6 h on Day -2 (before implant insertion), Day 6 (during the implant treatment period) and Day 13 (after implant removal) prior to or after implant insertion to characterize LH and FSH secretory patterns.

### 5.3.5 Analysis of Follicular Data

A follicular wave consisted of a follicle or a group of follicles that emerged and grew from 2 or 3 mm in diameter to =5mm (growth phase), before regressing to 2 or 3 mm in diameter (regression phase); time spent at =5mm was regarded as the static phase (Bartlewski et al., 1998; Duggavathi et al., 2003). The number of follicular waves, and the number of small follicles (=1 to =3 mm in diameter) per day were analyzed. Follicular data were integrated for both ovaries of each ewe.

### 5.3.6 Hormone Analysis

Estradiol (Joseph et al., 1992), Progesterone (Rawlings et al., 1984), LH (Rawlings et al., 1988) and FSH (Currie and Rawlings, 1989) concentrations were measured in serum samples by validated RIA procedures. The assay sensitivities (defined as the lowest concentration of a hormone capable of significantly displacing radio-labeled hormone from the antibody) were: 1.0 pg/mL for estradiol, 0.03 ng/mL for progesterone, and 0.1 ng/mL for LH and FSH. The ranges of standards were: 1.0 to 100 pg/mL, 0.1 to 5 ng/mL, 0.063 to 8.0 ng/mL and 0.12 to 16.0 ng/mL for the estradiol, progesterone, LH and FSH assays, respectively. A concentration equivalent to the sensitivity of the assay was assigned to serum samples with hormone concentrations lower than the assay sensitivity.

The intra- and inter-assay coefficients of variation (CVs) were 10.2% and 13.4% or 8.4% and 9.6% for reference sera with mean estradiol concentrations of 7.93 or 23.21 pg/mL, respectively. The intra- and inter-assay CVs were 11.8% and 14.7% or 6.9% and 9.4% for reference sera with mean progesterone concentrations of 0.28 or 1.08 ng/mL, respectively. The intra- and inter-assay CVs were 4.9% and 10.1% or 5.7% and 6.1% for reference sera with mean LH concentrations of 0.39 or 2.38 ng/mL, respectively.The intra- and inter-assay CVs were 4.8% and 6.2% or 4.3% and 5.8% for reference sera with mean FSH concentrations of 1.19 or 3.26 ng/mL, respectively.

The PC-PULSAR program (Gitzen and Ramirez, 1988) was used to assess mean and basal serum FSH and LH concentrations as well as FSH and LH pulse frequency and amplitude in blood samples collected every 12 min for 6h. The basal serum level ("smoothed series") was generated after the removal of short-term variation in hormone

concentrations, including possible pulses. Standard deviation criteria (G and Baxter parameters) were used for pulse detection.

Peaks of FSH in blood samples taken daily were identified using cycle-detection software (Clifton and Steiner, 1983). A fluctuation or cycle was defined as a progressive rise and fall in hormone concentrations that encapsulated a peak concentration (nadir-to-peak-to-nadir; Clifton and Steiner, 1983). Mean basal FSH concentrations were determined by averaging the lowest points between peaks (nadirs). Follicle stimulating hormone peak concentration was defined as the concentration of FSH observed at the apex of the FSH peak. Follicle stimulating hormone peak amplitude was defined as the difference between the FSH peak concentration and the nadir before the peak concentration. Follicle stimulating hormone peak duration was defined as the interval between the two nadirs encompassing the FSH peak.

**5.3.7 Statistical Analyses**

All data for hormone concentrations and ovarian follicles measured daily were normalized to the day of implant insertion both for presentation and statistical analyses. These data were analyzed for the full experimental period (i.e Days -5 to 21) or for the period of implant treatment (Days 0 to 12). Two-way repeated measures ANOVA (Sigma Stat 7 for Windows Version 2.03, 1997, SPSS Inc., Chicago, IL, USA) was used to assess differences in hormone concentrations and observations on ovarian follicles over time and among the groups of ewes (i.e., treated with estradiol only or estradiol plus progesterone implants). Two-way ANOVA was used to assess differences in LH and FSH secretory characteristics from blood samples collected every 12 min for 6 h (i.e., mean and basal concentrations, pulse frequency, and pulse amplitude) among groups of ewes and between intensive sampling days. Two-way ANOVA was also used

to assess differences in the number of FSH peaks and follicular waves over time and among groups of ewes. If the main effects, or their interactions, were significant ($P<0.05$), Fisher's protected least significant difference (LSD) was used as a post-ANOVA test to detect differences between individual means ($P<0.05$). Data are expressed as mean ± S.E.M.

## 5.4. RESULTS

### 5.4.1 Mean Daily Serum Estradiol Concentrations

During the period of treatment with implants, serum estradiol concentrations increased but the concentrations did not differ between ewes treated with estradiol alone compared to estradiol and progesterone releasing implants (10.93 ± 1.4 pg/mL vs. 8.00 ± 1.4 pg/mL; $P>0.05$; Figure 5.1).

### 5.4.2 Mean Daily Serum Progesterone Concentrations

During the period of treatment with implants, serum progesterone concentrations were greater in ewes treated with estradiol and progesterone releasing implants compared to ewes with implants releasing only estradiol (2.51 ± 0.6 ng/mL vs. 0.20 ± 0.04 ng/mL; $P<0.001$; Figure 5.2).

### 5.4.3 Characteristics of Serum LH Concentrations

Based on data from blood samples collected every 12 min for 6 hrs, mean and basal serum LH concentrations were at or close to assay sensitivity and did not differ amongst ewes treated with implants releasing estradiol alone or estradiol and progesterone or amongst days of frequent blood sampling ($P>0.05$). Sampling was done prior to, during and after the period of treatment with implants. Pulsatile LH secretion was abolished during treatment with implants ($P<0.05$).

### 5.4.4 Characteristics of Serum FSH concentrations

Based on blood samples collected daily, there were no differences in FSH peak concentrations, basal FSH concentrations and FSH peak duration amongst ewes treated with estradiol only and ewes treated with estradiol and progesterone implants ($P>0.05$; Table 5.1). However, during the implant treatment period, FSH peak amplitude was lower in ewes treated with implants releasing estradiol and progesterone compared to ewes treated with implants releasing only estradiol ($P<0.05$; Table 5.1).

Within the group of ewes treated with estradiol and progesterone releasing implants, the FSH peak amplitude was lower during the implant treatment period compared to the period before implant treatment and after implant removal ($P<0.05$; Table 5.1). For the ewes treated with estradiol and progesterone implants, the number of FSH peaks were greater after implant removal compared to the periods before and during implant treatment ($P<0.05$; Table 5.1).

Based on the blood samples collected daily, mean serum FSH concentrations did not differ between ewes with implants releasing only estradiol and implants releasing estradiol and progesterone ($P>0.05$; Table 5.1). However, in both groups of ewes, mean serum FSH concentrations were less during the implant treatment compared to before and after implant treatment. ($P<0.05$; Table 5.1).

Based on blood samples taken every 12 min for 6 hrs before (Day -2), during (Day 6) and after (Day 13) implant treatment, FSH secretory profiles were found to be non pulsatile by the PC-PULSAR program (Clifton and Steiner, 1983). In ewes treated with implants releasing only estradiol, mean serum FSH concentrations declined from Day -2 before implant insertion ($1.25 \pm 0.2$ ng/mL) to Day 6 of treatment ($0.96 \pm 0.1$

ng/mL; $P<0.05$). In ewes treated with both estradiol and progesterone releasing implants, mean serum FSH concentrations were lower on Day 6 ($0.93 \pm 0.1$ ng/mL) and Day 13 ($0.93 \pm 0.1$ ng/mL) compared to Day -2 ($1.26 \pm 0.2$ ng/mL; $P<0.05$).

### 5.4.5. Antral Follicle Development

No follicular waves emerged during implant treatment in both groups of ewes ($P<0.05$; Table 5.2). For the ewes treated with estradiol and progesterone releasing implants, the number of follicular waves emerging was greater after implant removal compared to before and during implant treatment ($P<0.05$; Table 5.2).

During the implant treatment period and as measured daily, there were no differences in the number of small follicles (=1 mm and =3 mm in diameter) amongst the ewes treated with implants releasing only estradiol or estradiol and progesterone ($P>0.05$; Table 5.2). For the ewes treated with estradiol and progesterone releasing implants, the number of small follicles was greater during implant treatment and after implant removal period compared to the period before implant treatment ($P<0.05$; Table 5.2).

Table 5.1: The number of FSH peaks, mean serum FSH concentrations and characteristics of FSH peaks (basal, peak concentration and amplitude, peak duration) during different periods of scanning (before, during and after implant treatment period) in anestrous Western White Face ewes treated for 12 d with silastic rubber implants (s.c.) containing 10% estradiol-17ß alone or in combination with silastic rubber implants (s.c.) containing 10% progesterone w/w.

| End points | Before implant treatment (Days -5 to -1) | | During implant treatment (Days 0 to 12) | | After implant removal (Days 13 to 21) | |
|---|---|---|---|---|---|---|
| | $E_2$ only implant | $E_2$ + $P_4$ implant | $E_2$ only implant | $E_2$ + $P_4$ implant | $E_2$ only implant | $E_2$ + $P_4$ implant |
| Mean number of FSH peaks | 1.40±0.2 | 1.40±0.3 $^{x}$ | 2.00±0.3 | 1.40±0.3 $^{x}$ | 1.80±0.2 | 2.40±0.4 $^{y}$ |
| Mean serum FSH concentrations (ng/mL) | 1.30±0.1 $^{x}$ | 1.29±0.1 $^{x}$ | 1.06±0.02 $^{y}$ | 0.91±0.1 $^{y}$ | 1.34±0.1 $^{x}$ | 1.12±0.1 $^{x}$ |
| Basal serum FSH concentrations (ng/mL) | 0.88±0.1 | 0.93±0.1 | 0.87±0.03 | 0.83±0.1 | 0.97±0.04 | 0.90±0.1 |
| FSH peak concentrations (ng/mL) | 1.73±0.2 | 1.53±0.1 | 1.44±0.1 | 1.25±0.1 | 1.51±0.1 | 1.36±0.1 |
| FSH peak amplitude (ng/mL) | 0.72±0.1 | 0.60±0.04 $^{x}$ | 0.64±0.1 $^{a}$ | 0.40±0.03 $^{by}$ | 0.63±0.04 | 0.57±0.1 $^{x}$ |
| FSH peak duration (days) | 4.00±0.4 | 3.50±0.2 | 3.57±0.1 | 4.60±0.7 | 3.70±0.4 | 3.47±0.2 |

Data are presented as mean±S.E.M.

$^{a, b}$ $P<0.05$; Different superscript letters within a row denote significant differences between the groups within each scanning period.

$^{x, y}$ $P<0.05$; Different superscript letters within a row denote significant differences between the periods of scanning within the groups.

Table 5.2: The number of follicular waves and mean number of small follicles (=1 mm to =3 mm in diameter) recorded daily during different periods of scanning (before, during and after implant treatment period) in anestrous Western White Face ewes treated for 12 d with silastic rubber implants (s.c.) containing 10% estradiol-17ß alone or in combination with silastic rubber implants (s.c.) containing 10% progesterone w/w.

| End points | Before implant treatment (Days -5 to -1) | | During implant treatment (Days 0 to 11) | | After implant removal (Days 12 to 21) | |
|---|---|---|---|---|---|---|
| | $E_2$ only implant | $E_2 + P_4$ implant | $E_2$ only implant | $E_2 + P_4$ implant | $E_2$ only implant | $E_2 + P_4$ implant |
| Mean number of follicular waves emerging | $1.20\pm0.2^{x}$ | $1.20\pm0.2^{x}$ | $0.00\pm0.0^{y}$ | $0.00\pm0.0^{y}$ | $1.80\pm0.2^{x}$ | $2.00\pm0.3^{z}$ |
| Mean number of small follicles | $24.45\pm2.4$ | $27.04\pm2.9^{x}$ | $27.12\pm2.3$ | $31.60\pm2.7^{y}$ | $26.81\pm2.5$ | $29.69\pm2.1^{y}$ |

Data are presented as mean ± S.E.M.

$^{a, b}$ $P<0.05$; Different superscript letters within a row denote significant differences between the groups within each scanning period.

$^{x, y, z}$ $P<0.05$; Different superscript letters within a row denote significant differences between the periods of scanning within the groups.

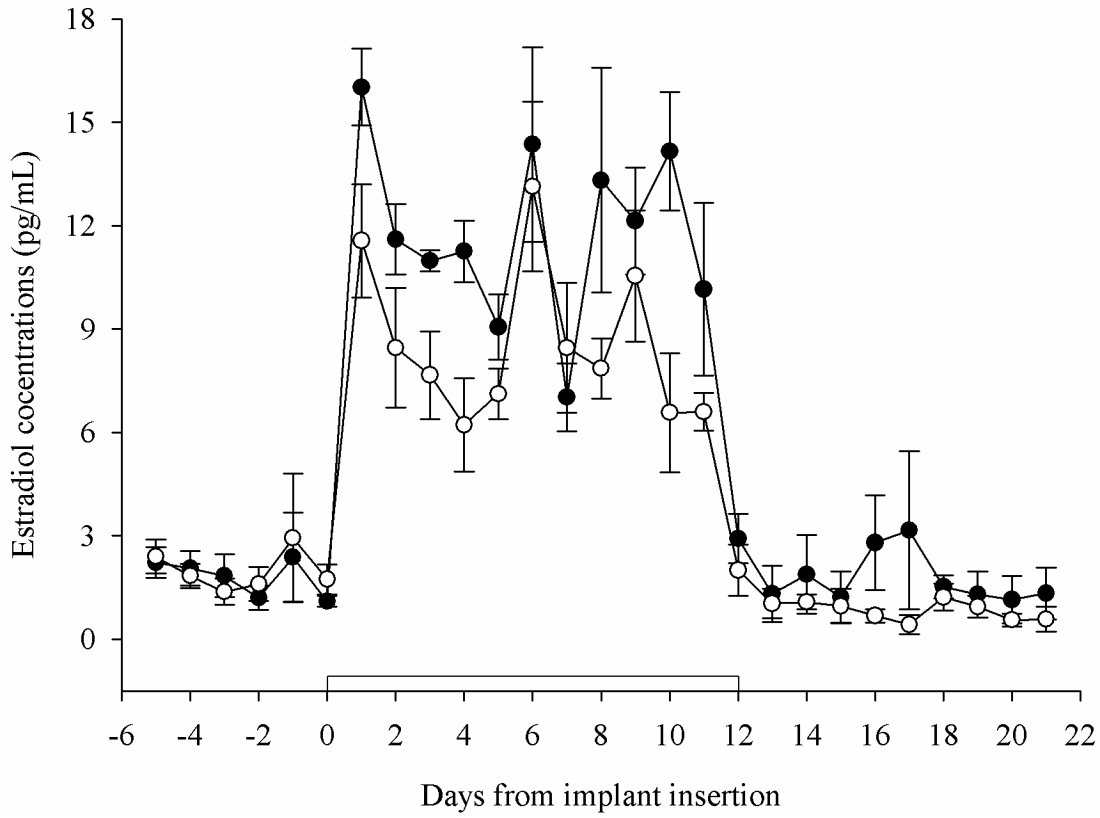


Fig. 5.1. Mean (±S.E.M.) daily serum estradiol concentrations over the entire experiment in anestrous Western White Face ewes treated for 12 d (Day 0-12; open rectangle on X-axis) with silastic rubber implants (s.c.) containing 10% estradiol-17ß (10 x 0.47 cm; black circles; n=5) alone or in combination with silastic rubber implants (s.c.) containing 10% progesterone w/w (11 x 0.48 cm; open circles; n=5). Data were normalized to the day of implant insertion (Day 0) in all ewes.

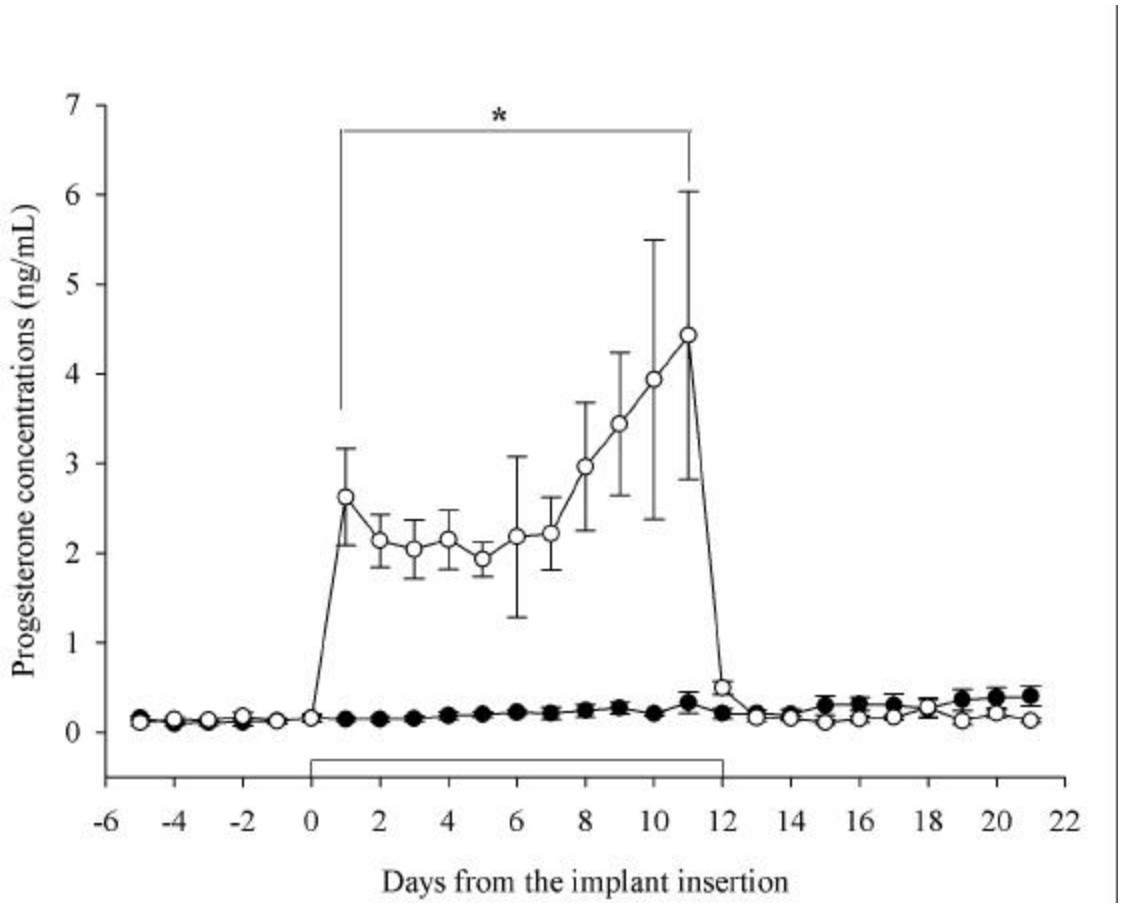


Fig. 5.2. Mean (±S.E.M.) daily serum progesterone concentrations over the entire experiment in anestrous Western White Face ewes treated for 12 d (Day 0-12; open rectangle on X-axis) with silastic rubber implants (s.c.) containing 10% estradiol-17ß (10 x 0.47 cm; black circles; n=5) alone or on combination with silastic rubber implants (s.c.) containing 10% progesterone w/w (11 x 0.48 cm; open circles; n=5). Data were normalized to the day of implant insertion (Day 0) in all ewes. Asterisks * and bar indicate the period over which ewes treated with estradiol alone implant differed from ewes treated with estradiol and progesterone ($P<0.001$).

## 5.5 DISCUSSION

In a previous study in anestrous ewes, treatment with implants releasing estradiol-17ß, that created serum concentrations of estradiol similar to the present study, had no effect on the number of FSH peaks, or peak concentration and amplitude. In contrast, in a previous study in cyclic ewes (Barrett et al., 2006), similar, supra-physiological concentrations of estradiol-17ß truncated the transient peaks in serum FSH concentrations that precede ovarian follicular waves. In the present study, supra-physiological serum concentrations of estradiol-17ß and physiological serum concentrations of progesterone, created in ewes treated with implants releasing estradiol-17ß and progesterone, suppressed the amplitude of the transient peaks in serum FSH concentrations. This suggests that progesterone enhances the inhibitory effects of estradiol on FSH secretion leading to a decrease in the amplitude of the peaks in serum FSH concentrations that precede and trigger follicular waves in anestrous ewes. The role of progesterone and estradiol in the negative feedback regulation of FSH secretion in the ewe is not as clear as that for LH and this is the first study of the combined role of these two steroids in regulating the FSH peaks that precede follicular waves in anestrous ewes. It is believed that the inhibitory effects of progesterone are more pronounced in seasonally anestrous ewes compared to ewes in the breeding season (Karsch et al., 1987).

Similar to the results of chapter 3 of this thesis, treatment with estradiol alone had no effects on basal serum concentrations of FSH, but mean serum FSH concentrations were suppressed. Giving progesterone with estradiol in the present study produced the same effect on mean serum FSH concentrations. The addition of

progesterone did not produce any decrease in basal serum concentrations of FSH. In another study in overiectomized ewes, treatment with progesterone alone had no effect on mean serum FSH concentrations (Hamernik et al., 1987), but when progesterone was given with estradiol means serum FSH concentrations were reduced (Moss et al., 1981). In the latter studies (Hamernik et al., 1987; Moss et al., 1981) basal serum concentrations of FSH or the concentration and amplitude of FSH peaks were not studied. Clearly, the only additive effect of giving progesterone and estradiol to anestrous ewes was to reduce the amplitude of the peaks in serum FSH concentrations that precede follicular waves.

The increase in the number of FSH peaks and follicular waves after implant removal in the present study, in ewes given implants releasing estradiol-17ß and progesterone, may be partly due to the removal of a synergistic inhibitory effect by estradiol and progesterone on FSH secretory peaks.

In the present study, supra-physiological concentrations of estradiol-17ß alone or in combination with progesterone abolished LH pulses. It is intriguing that in our previous study in cyclic ewes (Barrett et al., 2006) supra-physiological concentrations of estradiol-17ß had no significant effect on the LH secretory pattern. There is a higher threshold for suppression of LH secretory pulses by estradiol-17ß in cyclic ewes compared to anestrous ewes (Goodman and Karsch, 1980; Joseph et al., 1992; Martin et al., 1983).

In the present study, one of the most profound effects of the implants releasing steroids was on follicle development. In ewes given implants releasing estradiol-17ß alone, follicular wave emergence was blocked with no effect on the amplitude or

number of FSH peaks. Because LH pulses were suppressed, follicles failed to receive the appropriate LH support and had difficulty growing beyond 2 mm in diameter, even though FSH peaks were seen. In the present study, the implants releasing only estradiol-17ß had no effect on the number of follicles in the small follicle pool (= 1 mm to =3 mm in diameter), as was seen in a previous study in anestrous ewes (chapter 2 of this thesis). Treatment of cyclic ewes with implants releasing estradiol and progesterone decreased the amplitude of FSH peaks and reduced the small follicle pool in the ovary (Barrett et al., 2007). It was concluded that FSH peaks are required to maintain the small follicle in the cyclic ewe (Barrett et al., 2007). In the present study, Supra-physiological concentrations of estradiol- 17ß and physiological concentrations of progesterone together decreased FSH peak amplitude, blocked follicular waves but resulted in an increase in the number of follicles in the small follicle pool. The effect of FSH peak concentration and amplitude in maintenance of the small follicle pool in the ewe is obviously not clear.

Similar to previous studies (Barrett et al., 2006; Barrett et al., 2007), another interesting observation from the present study is that FSH peaks continued to occur in the absence of follicle wave development during the implant treatment period. The peaks were objectively identified by the cycle detection software (Clifton and Steiner, 1983). In cattle in particular (Ginther et al., 2002), it is believed that the waxing and waning of secretion of various products of dominant ovarian follicles regulate the periodicity of follicular waves. Based on the present results, it appears that the FSH peaks occur independently of the production of any ovarian follicular secretory products in anestrous ewes. FSH peaks were not regulated by estradiol as constant serum

concentrations were maintained by the implant treatment. Another possible ovarian regulator of FSH peaks in the anestrous ewe would be inhibin (Martin et al., 1988). Unlike estradiol, which is produced by only large estrogenic follicles, inhibin is produced from a wider range of follicles (Bjersing et al., 1972). However, serum concentrations of inhibin do not fluctuate with the rhythm of follicular waves in anestrous ewes (Evans et al, 2001a). The present and previous findings suggest that there could be some mechanisms other than ovarian follicular feedback that regulate the timing of the FSH peaks proceeding follicle wave emergence in the ewe.

In summary, supra-physiological concentrations of estradiol suppressed follicle wave development by abolishing LH pulses in anestrous ewes; the FSH secretory peaks that precede follicular waves were not affected. Supra-physiological concentrations of estradiol-17ß with physiological concentrations of progesterone, decreased FSH peak amplitude and abolished LH pulses. Treatment with implants releasing estradiol and progesterone increased the small follicle pool (=1 mm to =3 mm in diameter), in contrast to cyclic ewes where a similar treatment decreased the small follicle pool. The involvement of secretory products from the follicles of a wave in regulating the occurrence of the FSH peak that initiate the subsequent follicular wave in the ewe appears to be questionable.

# Chapter 6: PULSED GnRH SECRETION AND THE FSH SECRETORY PEAKS THAT INITIATE OVARIAN ANTRAL FOLLICULAR WAVE EMERGENCE IN ANESTROUS EWES [*]

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## 6.1 ABSTRACT

In the ewe, immunization against GnRH blocks LH pulses but mean serum FSH concentrations are only partly reduced; the fate of the FSH peaks that precede ovarian follicular waves has not been studied. In this study, we used immunization against GnRH to examine the need for pulsed GnRH secretion in the genesis of FSH peaks in the anestrous ewe. Six anestrous ewes were given a GnRH immunogen on Day 0 and a booster injection on Day 28. Control ewes (n=6) received adjuvant only. Transrectal ultrasonography was performed daily for 2 d prior to and 10 d following both the primary (Days -2 to 10) and booster (Days 26 to 38) injections and for a 13 day period beginning 26 d after booster injection (Days 54 to 66). Blood samples were collected daily. Intensive bleeding (every 12 min for 7 h) was performed on Days 9, 37, and 65 of the experimental period to characterize the pulsatile pattern of LH secretion. GnRH antibody titres were increased and LH pulses were abolished immediately after booster immunization ($P<0.05$). The number of FSH peaks, FSH peak concentration and amplitude and basal FSH concentrations were only decreased in immunized ewes in the period of observations starting 26 d after booster immunization ($P<0.05$); however, some peaks were still seen. The number of follicular waves was decreased in the period around booster immunization and no follicular waves were seen during the period starting 26 d after booster immunization in immunized ewes ($P<0.05$). In summary, in

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anestrous ewes, when pulsed LH secretion was abolished by immunization against GnRH, the peaks in serum concentrations of FSH that trigger ovarian follicular waves continued for a period of time. We concluded that although blocking the effects of GnRH gradually causes a diminution of FSH secretion, there is no acute requirement for GnRH in the regulation of FSH peaks. The existence of FSH peaks in the absence of follicular waves, and pulsed LH secretion, suggests that some endogenous rhythm may drive the occurrence of FSH peaks, independent of both changes in negative feedback by secretory products from ovarian antral follicles and GnRH.

## 6.2 INTRODUCTION

In the ewe, both during the breeding (Bartlewski et al., 1999a; Evans et al., 2000; Ginther et al., 1995; Schrick et al., 1993) and anestrous (Bartlewski et al., 1998; Souza et al., 1996) seasons, ovarian antral follicles emerge or grow in a wave like pattern. One or more follicles grow from a pool of small follicles (= 2 mm to = 3 mm in diameter) every 3-5 d, to reach a diameter of = 5 mm before ovulation or regression. Each follicular wave is preceded by a peak in serum concentrations of FSH lasting 3 to 4 d; this peak has been shown to be an essential trigger for the emergence of a follicular wave (Bartlewski et al., 1999a; Bartlewski et al., 1998; Duggavathi et al., 2003; Evans et al., 2000; Ginther et al., 1995; Souza et al., 1998).

LH secretion in the ewe is clearly pulsatile with approximately 1 to 6 pulses per 6 h (Baird, 1978; Karsch et al., 1979; Karsch et al., 1980) and driven by pulsatile secretion of GnRH (Clarke and Cummins, 1982; Levine et al., 1982). Pulses of FSH secretion can be seen in hypophyseal portal blood in the adult ewe but the major secretion of FSH is a continuous basal constitutive secretion; pulses in serum

concentrations of FSH are not seen in the peripheral circulation in intact adult ewes (McNeilly, 1988; Padmanabhan et al., 2003; Padmanabhan et al., 1997; Padmanabhan and Sharma, 2001; Wallace and McNeilly, 1986). Active immunization of ewes against LHRH and treatments with GnRH agonists and antagonists decreased or abolished pulsed LH secretion in the ewe, whereas serum concentrations of FSH were either not altered or partially suppressed (Clarke et al., 1998; Mariana et al., 1998; McNeilly and Fraser, 1987) (Campbell et al., 1997; Campbell et al., 1998; Padmanabhan et al., 2003). In the studies described above, the FSH peaks that precede follicular wave emergence were not recognized or studied. We do not know if the partial suppression of serum concentrations of FSH that was seen when the effects of GnRH were blocked involved inhibition of the peaks in FSH secretion that cause follicular wave emergence. In cattle, the regulation of the peaks in FSH secretion that precede follicular waves appears to involve changes in negative feedback by secretory products from ovarian antral follicles as they grow and regress (Driancourt, 2001; Ginther et al., 1996). The most obvious ovarian products to be involved would be inhibin and estradiol (Beg et al., 2002; Mihm and Evans, 2008). Changes in negative feedback by ovarian secretory products may not regulate the sequence of FSH peaks in sheep as the peaks appear to occur in the absence of follicular waves and in ovariectomized ewes (Barrett et al., 2006; Duggavathi et al., 2005a; Duggavathi et al., 2008).

In cyclic ewes, treatment with estradiol releasing implants blocked follicular waves but FSH peaks of reduced amplitude continued to occur at the expected frequency (Barrett et al., 2006). Secretory patterns of inhibin are not closely correlated with the pattern of follicular waves and FSH secretion in cyclic ewes (Souza et al.,

1998; Souza et al., 1997). In anestrous ewes, regular follicular waves continue as well as the preceding peaks in FSH secretion but correlated changes in serum concentrations of estradiol and inhibin-A are not seen (Bartlewski et al., 1998; Evans et al, 2001a; Souza et al., 1996). A GnRH-independent component of episodic FSH release has been noted in the luteal phase of the ewe (Padmanabhan et al., 2003; Padmanabhan and Sharma, 2001; Van Cleeff et al., 1995), and a separate FSH releasing hormone has been suggested (McCann et al., 1998; McCann et al., 1983; Padmanabhan and McNeilly, 2001; Padmanabhan and Sharma, 2001).

Our primary objective was to investigate the GnRH dependence of the peaks in serum FSH secretion that precede and trigger ovarian follicular waves in the anestrous ewe using immunization against GnRH. We hypothesized that immunization against GnRH would block the pulsatile secretion of LH but allow continued peaks in FSH secretion in anestrous ewes.

## 6.3 MATERIALS AND METHODS

### 6.3.1 Animals

Care and handling of experimental animals were done according to the Canadian Council on Animal Care's published guidelines. Sexually mature, clinically healthy, seasonally anestrous, WWF ewes were kept outdoors in sheltered paddocks. Ewes were fed a maintenance diet of hay; cobalt-iodized saltlicks and water were freely available. The WWF is a cross between the Columbia and Rambouillet breeds.

### 6.3.2 Ultrasound Technique

Ovarian antral follicular dynamics were monitored in all ewes by transrectal ovarian ultrasonography (scanning) using a 7.5-MHz linear transducer stiffened with a hollow plastic rod and connected to a B-mode, real-time echo camera (Aloka SSD-900, Overseas Monitor, Richmond, BC, Canada). This technique has been validated for monitoring ovarian follicular dynamics and CL detection in sheep (Duggavathi et al., 2003; Ravindra et al., 1994; Schrick et al., 1993). All images were viewed at a magnification of X 1.5 with constant gain and focal point settings. Ovarian images were recorded (Panasonic AG 1978, Matsushita Electric, Mississauga, ON, Canada) on high-grade video tapes (Fuji S-VHS, ST-120 N, Fujifilm, Tokyo, Japan) for later examination. The relative position and dimension of follicles and luteal structures were also sketched on ovarian charts.

### 6.3.3 Experimental Design

The experimental design is summarized in Figure 1. Twelve ewes were used in this study. Six ewes were injected with a GnRH immunogen mixture [ovalbumin–Luteinizing hormone releasing hormone (LHRH)-7 (OL) and thioredoxin–LHRH-7 (TL) recombinant proteins (Quesnell et al., 2000; Zhang et al., 1999)] in the axillary region (sc). A primary injection was given on Day 0 and a booster injection was given 4 weeks later (Day 28). Control ewes (n=6) were injected with the same adjuvant used to prepare the vaccine. Briefly, the OL and TL proteins were produced from previously constructed OL and TL genes generated by recombinant DNA techniques and as previously described (Quesnell et al., 2000; Zhang et al., 1999). Recombinant OL and TL genes were over expressed in *E. coli.* Affinity chromatography utilizing a $Ni^{2+}$

column was used to purify the OL and TL proteins by binding to histidine residues of the proteins. Equimolar amounts of the OL and TL proteins (10 nM), totalling 1.0 mg of protein, were suspended in 1.0 mL of 6 M urea and emulsified in 1.0 ml of modified complete Freund's adjuvant (Calbiochem, San Diego, CA, USA) for the primary immunization. Modified incomplete Freund's adjuvant (Sigma, St Louis, MO, USA) was used for the booster injection. Immunizations were distributed over four subcutaneous sites in the axillary region. Transrectal ultrasonography was performed daily for 2 d prior to and 10 d following both the primary (Days -2 to 10) and booster injections (Days 26 to 38) and for a 13 day period beginning 26 d after the booster injection (Days 54 to 66). Blood samples were collected daily through the three periods of scanning and on Days 134 and 160 after primary immunization. Intensive blood sampling (every 12 min for 7 h) was performed on Days 9, 37 and 65 of the experimental period to characterize the pattern of pulsatile LH secretion.

**6.3.4 Analysis of Follicular Data**

A follicular wave consists of 1 to 3 follicles that emerge and grow from the pool of small follicles in the size range of 2 or 3 mm in diameter. These follicles grow to = 5mm (growth phase), before regressing back to 2 or 3 mm in diameter (regression phase); time spent at = 5mm is regarded as the static phase (Bartlewski et al., 1998). The numbers of follicular waves, the number of follicles in various size categories recorded daily and daily maximum follicle diameter were analyzed. Follicular data were integrated for both ovaries of each ewe.

### 6.3.5 Blood Sampling and Hormone Analysis

Blood samples (10 mL) taken daily were collected by jugular venipuncture into vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA). For intensive sampling, blood was collected via indwelling jugular catheters (5 ml/sample; vinyl tubing, 1.0 mm i.d x 1.5 mm o.d; SV70, Critchley Electrical Products Pty Ltd., Auburn, NSW, Australia). All samples were permitted to clot at room temperature for 18 to 24 h. Samples were then centrifuged for 10 min at 1500 x g, and serum was removed and kept at !20 $^{o}$C until assayed.

GnRH antibody titres were analyzed for the first and the last but one sample of each period of scanning and samples collected on Days 134 and 160 after primary immunization. GnRH antibody titres were determined using a radioimmunoassay procedure described previously (Finnerty et al., 1994). Briefly, 100 µl $^{125}$I-labelled GnRH (12000 dpm; Amersham International Limited, Amersham, UK) was added to 400 µl aliquots of each five serial dilutions of serum (1:40 to 1:640). After an overnight incubation at 4 $^{o}$C, 500 µl of 25% polyethylene glycol (PEG; Sigma, St Louis, MO, USA) was added to each tube. The tubes were vortexed, incubated at room temperature for a further 30 min and then centrifuged at 3000 g for 20 min. The pellet was resuspended in 500 µl of 12.5% PEG solution. The tubes were again vortexed and incubated at room temperature for 20 min before centrifugation at 3000 g for 10 min. The supernatant was poured off and the radioactivity in the pellet was measured in a gamma counter. The results are presented as the percentage of total $^{125}$I GnRH bound at a serum dilution of 1:160 (Figure 6.2). This dilution gave the clearest trend in titre with time.

LH (Rawlings et al., 1988), FSH (Currie and Rawlings, 1989) and Estradiol (Joseph et al., 1992) concentrations were measured in serum samples by validated radioimmunoassay procedures. The assay sensitivities (defined as the lowest concentration of a hormone capable of significantly displacing radio-labeled hormone from the antibody) were 0.1 ng/mL for LH and FSH and 1.0 pg/mL for estradiol. The ranges of standards were 0.06 to 8.0 ng/mL, 0.12 to 16.0 ng/mL, and 1.0 to 100 pg/mL, in the LH, FSH and estradiol assays, respectively. A concentration equivalent to the sensitivity of the assay was assigned to serum samples with hormone concentrations lower than the assay sensitivity. Serum samples collected daily during the periods of scanning were analyzed for concentrations of FSH and estradiol. All serum samples collected every 12 min were analyzed for concentrations of LH.

The intra- and inter-assay CVs were 7.3% and 9.4% or 6.1% and 5.8% for reference sera with mean LH concentrations of 0.40 or 2.12 ng/mL, respectively. The intra- and inter-assay CVs were 6.8% and 10.6% or 2.5% and 2.6% for reference sera with mean FSH concentrations of 0.61 or 3.53 ng/mL, respectively. The intra- and inter-assay coefficients of variation were 9.5% and 13.5% or 7.5% and 9.2% for reference sera with mean estradiol concentrations of 8.48 or 22.66 pg/mL, respectively.

The PC-PULSAR program (Gitzen and Ramirez, 1988) was used to assess mean and basal serum LH concentrations as well as LH pulse frequency and amplitude in blood samples collected every 12 min for 6 h. Peaks of FSH in blood samples taken daily were identified using cycle-detection software (Clifton and Steiner, 1983). A fluctuation or cycle was defined as a progressive rise and fall in hormone concentrations that encapsulated a peak concentration (nadir-to-peak-to-nadir; Clifton and Steiner,

1983). Mean basal FSH concentrations were determined by averaging the lowest points between peaks (nadirs). Follicle stimulating hormone peak concentration was defined as the concentration of FSH observed at the apex of the FSH peak. Follicle stimulating hormone peak amplitude was defined as the difference between the FSH peak concentration and the nadir before the peak concentration.

### 6.3.6 Statistical Analyses

Hormone concentrations in blood samples collected daily and numbers of follicles in various size classes observed daily were normalized to the day of primary immunization (Day 0) for analyses and presentation. Two-way repeated measures Analysis of Variance (RM ANOVA; Sigma Stat 7 for Windows Version 2.03, 1997, SPSS Inc., Chicago, IL, USA) was used to assess differences in hormone concentrations and numbers of follicles in size classes amongst periods of scanning (Days -2 to 10, Days 26 to 28 and Days 54 to 66) and groups of ewes (i.e., with or without GnRH immunogen). Two-way RM ANOVA was also used to assess differences in LH secretory characteristics from blood samples collected every 12 min for 7 h (i.e., mean and basal concentrations, pulse frequency, and pulse amplitude) amongst immunized and control ewes and between days of intensive sampling (Days 9, 37 and 65). Two-way RM ANOVA was used to assess differences in characteristics of FSH peaks (FSH peak concentration and amplitude and basal FSH concentrations) and the number of FSH peaks and follicular waves amongst treated and control ewes and periods of scanning. If the main effects, or their interactions, were significant ($P<0.05$), Fisher's protected least significant difference (LSD) was used as a post-ANOVA test to detect differences between individual means ($P<0.05$). Data are expressed as mean ± S.E.M.

## 6.4 RESULTS

### 6.4.1 GnRH Antibody titre

There was an increase in antibody titre after the booster immunization given on Day 28 and titres remained high out to Day 160 after primary immunization (Figure 6.2).

### 6.4.2 Characteristics of Serum LH Concentrations

No LH pulses were seen at the intensive bleeds on Days 37 and 65 after primary immunization (9 and 37 d after booster immunization) and mean serum LH concentrations were decreased in immunized compared to control ewes ($P<0.001$; Figure 6.3). Basal serum LH concentrations was not affected by the immunization ($P>0.05$; Figure 3).

### 6.4.3 Characteristics of Serum FSH concentrations

Based on blood samples collected during each scanning period, and as identified by the cycle detection software, the number of FSH peaks, FSH peak concentration and amplitude and basal serum FSH concentrations were decreased in immunized compared to control ewes during Days 54 to 66 after primary immunization (26 to 38 d after booster immunization; $P<0.05$; Figure 6.4A, 6.4B, 6.4C and 6.4D). However, there was no differences for any of the parameters of FSH secretion above amongst immunized and control ewes during Days -2 to 10 and Days 26 to 38 after primary immunization (-2 to 10 d after booster immunization; $P>0.05$; Figure 6.4A, 6.4B, 6.4C and 6.4D). Within the immunized ewes, the FSH peak concentrations, amplitude and number of FSH peaks were decreased during Days 54 to 66 compared to Days -2 to 10 and Days 26 to 38 after primary immunization ($P<0.05$; Figure 6.4A, 6.4B and 6.4D); however,

the basal FSH concentrations were only decreased during Days 54 to 66 compared to only Days 26 to 38 ($P<0.05$; Figure 6.4C).

### 6.4.4 Mean Daily Serum Estradiol Concentrations

Serum estradiol concentrations did not differ between immunized and control ewes during any of the scanning periods ($P>0.05$; Figure 6.5); however, concentrations increased over time in both groups of ewes ($P<0.05$; Figure 6.5).

### 6.4.5 Antral Follicle Development

There were fewer follicular waves emerging per ewe during Days 26 to 38 (-2 to 10 d after booster immunization) and no follicular waves emerging per ewe during Days 54 to 66 after primary immunization (26 to 38 d after booster immunization) in immunized compared to control ewes ($P<0.05$; Table 6.1). There was no difference in the number of small (=1mm and =3mm in diameter) follicles amongst the scanning periods or groups ($P>0.05$; Table 6.1). The number of medium (4 mm in diameter) follicles was decreased in immunized ewes compared to control ewes during Days 54 to 66 ($P<0.05$; Table 6.1). Within the immunized ewes, the number of medium sized follicles was less during Days 54 to 66 compared to Days -2 to 10 and Days 26 to 38 after primary immunization ($P<0.05$; Table 6.1). The number of large follicles (=5mm in diameter) were decreased in immunized compared to control ewes during Days 54 to 66 after primary immunization ($P<0.05$; Table 6.1). The maximum follicle diameter was decreased in immunized compared to control ewes during Days 26 to 38 and Days 54 to 66 after primary immunization ($P<0.05$; Table 6.1). Within the immunized ewes, the number of follicular waves, large sized follicles and maximum follicle diameter were decreased during Days 54 to 66 compared to Days -2 to 10 and Days 26 to 38 and also

decreased during Days 26 to 38 compared to Days -2 to 10 after primary immunization ($P<0.05$; Table 6.1).

Table 6.1: The number of follicular waves, the number of follicles in various size categories recorded daily and daily maximum follicle diameter during each period of scanning in anestrous Western White Face ewes treated with a GnRH immunogen (treatment) or the adjuvant used to prepare the vaccine (control) on Day 0 (primary immunization) and Day 28 (booster immunization).

| End points | Scanning period 1 (Days -2 to 10) | | Scanning period 2 (Days 26 to 38) | | Scanning period 3 (Days 54 to 66) | |
|---|---|---|---|---|---|---|
| | Control | Treatment | Control | Treatment | Control | Treatment |
| Mean number of Follicular Waves | 3.17±0.3 | 3.0±0.3 $^{x}$ | 3.33±0.2 $^{a}$ | 2.0±0.4 $^{bx}$ | 2.67±0.2 $^{a}$ | 0.00±0.0 $^{by}$ |
| Mean number of small follicles (=1mm and =3mm in diameter) | 24.74±1.9 | 22.97±2.1 | 24.59±2.3 | 23.29±2.0 | 21.74±1.6 | 21.54±1.4 |
| Mean number of medium follicles (4 mm in diameter) | 1.69±0.5 | 1.86±0.4 $^{x}$ | 1.91±0.5 | 2.0±0.5 $^{x}$ | 1.63±0.6 $^{a}$ | 0.0±0.0 $^{by}$ |
| Mean number of large follicles (=5mm in diameter) | 1.80±0.4 | 2.31±0.4 $^{x}$ | 1.97±0.5 | 1.41±0.4 $^{y}$ | 2.06±0.4 $^{a}$ | 0.0±0.0 $^{bz}$ |
| Mean daily maximum follicle diameter (mm) | 5.00±0.2 | 5.06±0.0 $^{x}$ | 5.01±0.1 $^{a}$ | 4.64±0.2 $^{by}$ | 5.03±0.1 $^{a}$ | 2.58±0.2 $^{bz}$ |

Data are presented as mean ± S.E.M.

$^{a, b}$ $P<0.05$; Different superscript letters within a row denote significant differences between the groups within each scanning period.

$^{x, y, z}$ $P<0.05$; Different superscript letters within a row denote significant differences between the periods of scanning within the groups.

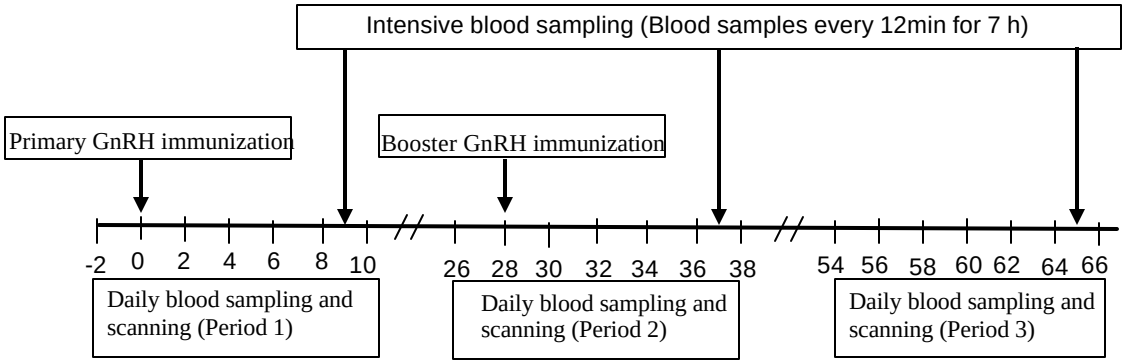


Fig. 6.1. Schematic representation of the experimental design used in the present study. Six anestrous Western White Face ewes received injections of a GnRH immunogen mixture (2 ml; sc) on Day 0 (Primary) and Day 28 (Booster). Six control ewes received the adjuvant used to prepare the vaccine. Daily transrectal ovarian ultrasonography and blood sampling was performed on all ewes on Days -2 to 10, 26 to 38 and 54 to 66 after primary immunization. Blood samples were also taken every 12 min for 7 h (intensive bleeds) on Day 9, Day 37 and Day 65 after primary immunization.

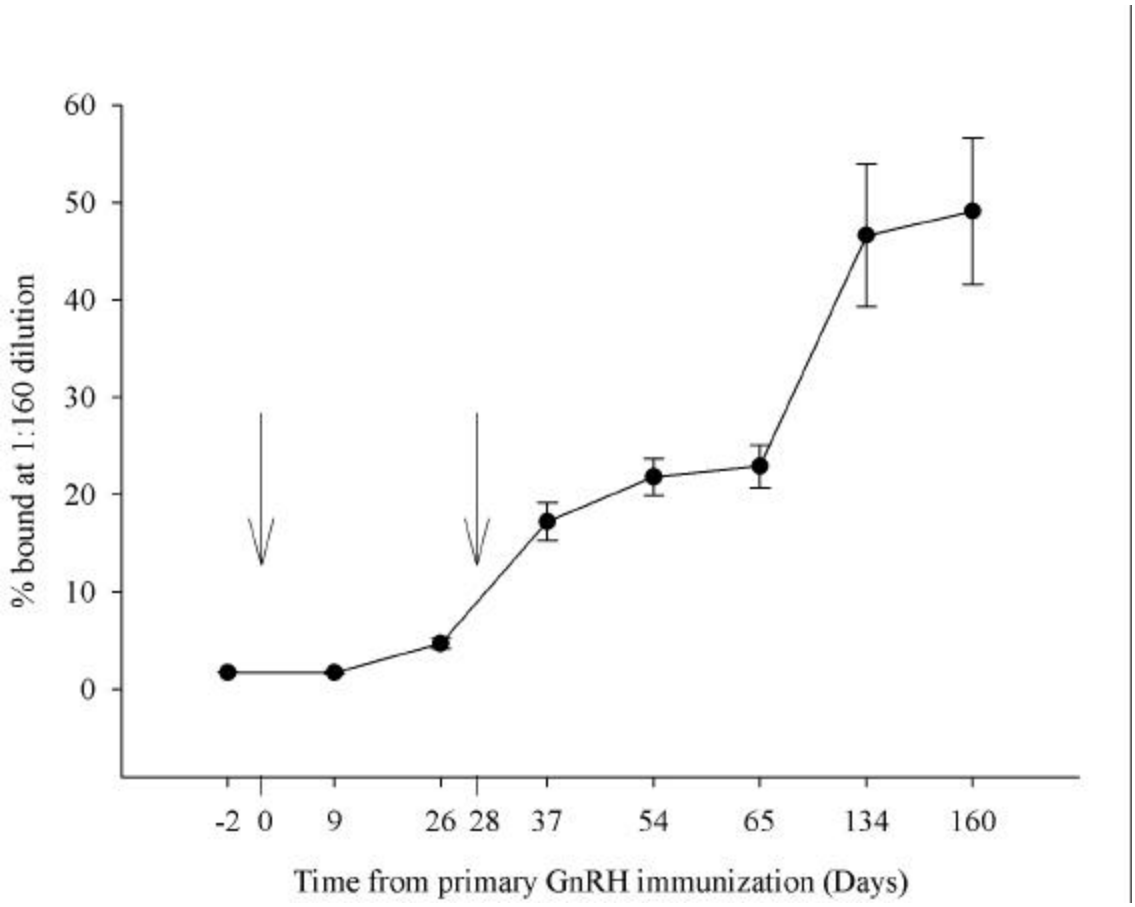


Fig. 6.2. Mean (± S. E. M) antibody binding to GnRH expressed as percentage bound at a 1:160 dilution in anestrous Western White Face ewes injected with a GnRH immunogen mixture on Day 0 (primary) and Day 28 (Booster). Arrows represent the days of primary and booster immunizations.

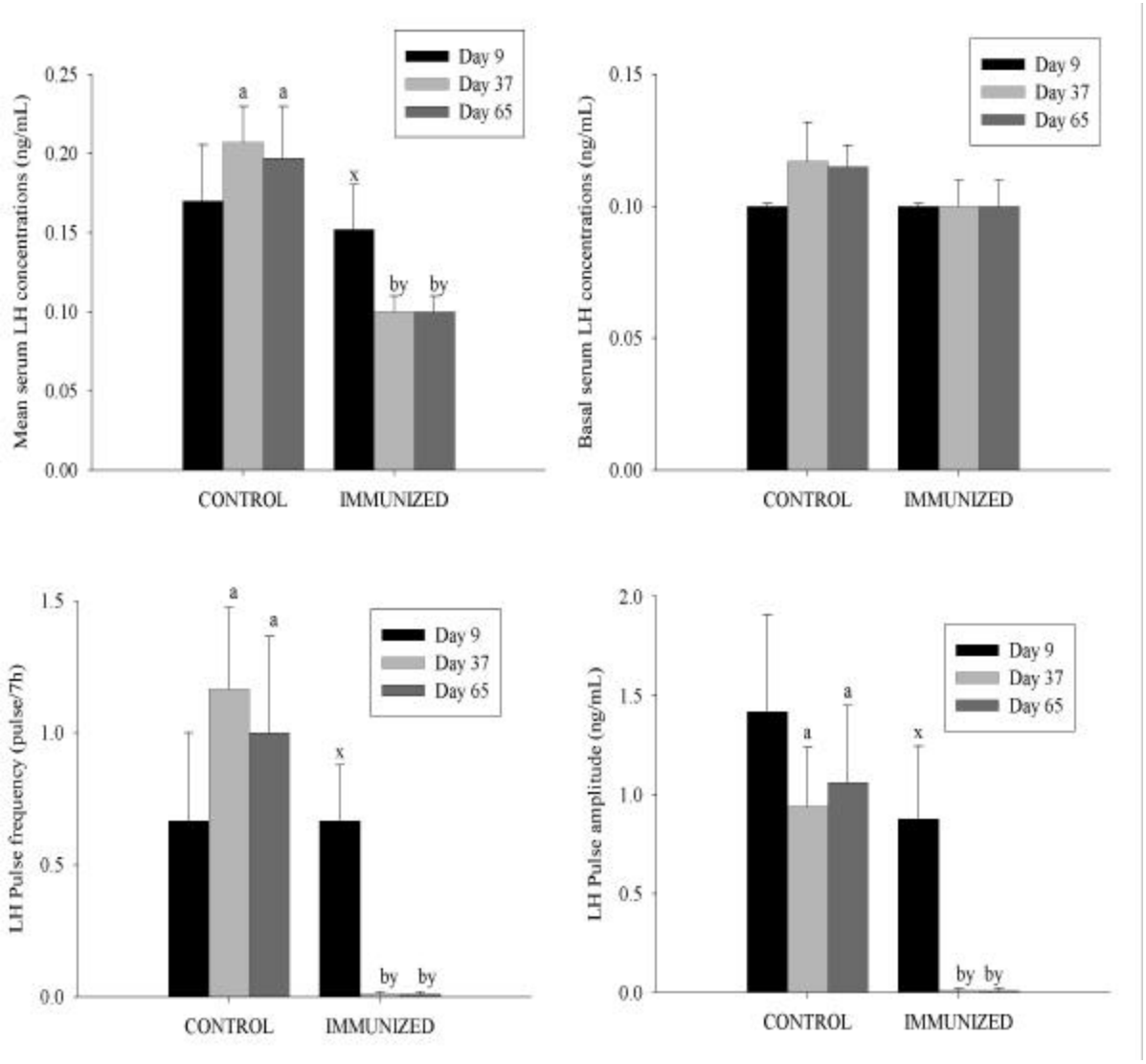


Fig. 6.3. The characteristics of pulsatile LH secretion (mean and basal serum LH concentrations and LH pulse frequency and amplitude; mean ± S.E.M) determined from serum samples collected every 12 min for 7 h on Day 9 (black bars), Day 37 (light gray bars) and Day 65 (dark grey bars) after initial GnRH immunization in anestrous Western White Face ewes. Treatment ewes received injections of a GnRH immunogen mixture (2 ml; sc) on Day 0 (Primary) and Day 28 (Booster). Control ewes received the adjuvant used to prepare the vaccine. Letters (a-b) indicate differences between control ewes and ewes treated with GnRH immunogen ($P<0.001$) within the respective intensive sampling period. Letters (x-y) indicate differences between the days of intensive blood sampling ($P<0.001$) for control ewes or ewes treated with GnRH.immunogen [n=6 (control), n=6 (treatment)].

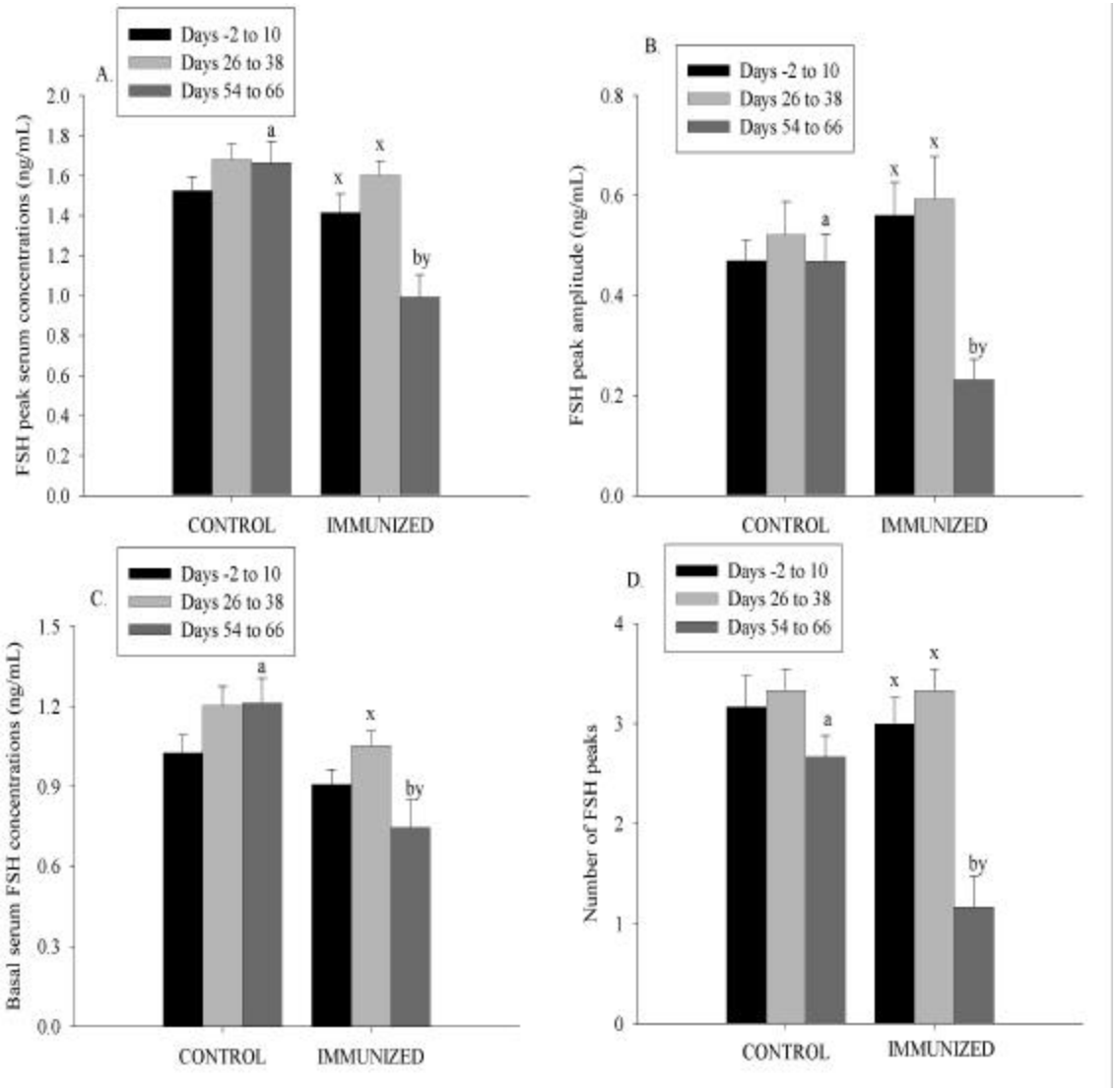


Fig. 6.4. The characteristics of FSH secretion (FSH peak concentration and amplitude and basal serum FSH concentrations; mean ± S.E.M) and number of FSH peaks (mean ± S.E.M) determined from serum samples collected daily during Days -2 to 10 (black bars), 26 to 38 (light gray bars) and 54 to 66 (dark grey bars) after initial GnRH immunization in anestrous Western White Face ewes. Treatment ewes received injections of GnRH immunogen mixture (2 ml; sc) on Day 0 (Primary) and Day 28 (Booster). Control ewes received the adjuvant used to prepare the vaccine. Letters (a-b) indicate differences between control ewes and ewes treated with GnRH immunogen ($P<0.001$) within the respective intensive sampling period. Letters (x-y) indicate differences between the days of intensive blood sampling ($P<0.001$) for control ewes or ewes treated with GnRH.immunogen [n=6 (control), n=6 (treatment)].

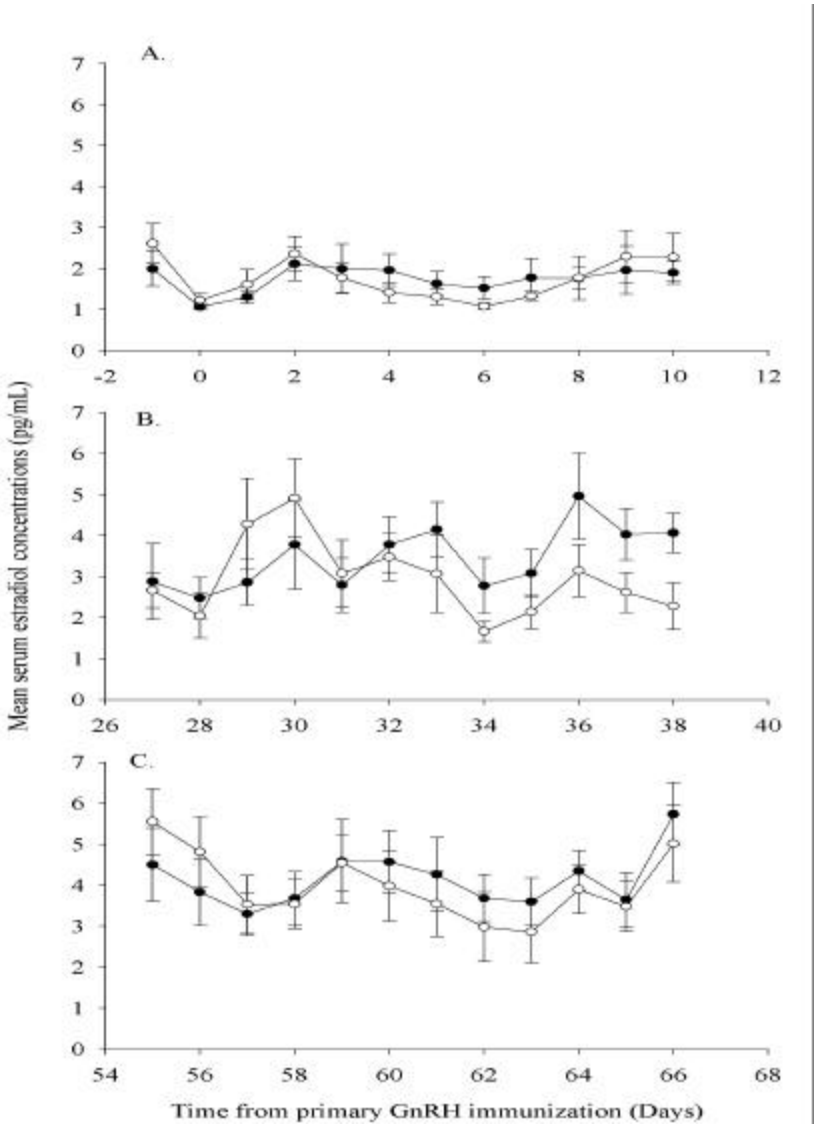


Fig. 6.5. Mean (±S.E.M.) daily serum estradiol concentrations during Days -2 to 10 (panel A), 26 to 38 (Panel B) and 54 to 66 (Panel C) in anestrous Western White Face ewes treated with a GnRH immunogen mixture (2 mL; sc; open circles; n=6) or adjuvant used to prepare the vaccine (black circles; n=6) on Day 0 (primary) and Day 28 (booster). Data were normalized to the day of initial immunzation (Day 0) in all ewes.

## 6.5 DISCUSSION

In the present study, pulsatile LH secretion was abolished following the booster immunization at Day 28 after the initial injection of immunogen, coincident with the appearance of anti-GnRH antibodies. However, the peaks of FSH secretion that herald ovarian follicular waves were not reduced in number and size until Days 54 to 66 after primary immunization (26 to 38 d after booster immunization). In previous studies which involved treatment of ewes with a GnRH agonist (McNeilly and Fraser, 1987) or antagonist (Campbell et al., 1997; Campbell et al., 1998; Padmanabhan et al., 2003), or active immunization (Clarke et al., 1998; Mariana et al., 1998) against GnRH, there was a consistent suppressive effect on pulsatile LH secretion. However, when FSH was studied, suppression of FSH secretion was not achieved or only partial suppression was noted (Padmanabhan et al., 2003; Padmanabhan and McNeilly, 2001). In the studies indicated above, only mean serum FSH concentrations were examined; the dependence of secretion of the FSH peaks that precede follicular waves on GnRH secretion was not studied. Clearly, based on the results of the present study, the peaks in FSH secretion that precede follicular waves can continue for several weeks even in the presence of anti-GnRH antibodies of sufficient titre to block pulsed LH secretion; however, peak concentration and amplitude and intervening basal serum FSH concentrations are affected by anti-GnRH antibodies 4 weeks after the booster immunization. The partial but immediate suppression of mean serum concentrations of FSH after immunization against GnRH or treatment with GnRH agonists or antagonists, recorded in the literature discussed above, would not appear to involve an immediate suppression of FSH peaks based on the present observations. When FSH concentrations in pituitary

portal blood were measured in previous studies, pulses of FSH secretion and the frequency of LH pulses were noted but the major modality of FSH secretion appeared to be reflected in high basal serum concentrations of FSH (McNeilly, 1988; Padmanabhan et al., 2003; Padmanabhan et al., 1997; Padmanabhan and Sharma, 2001; Wallace and McNeilly, 1986). It is therefore, unclear as to what regulates the peaks in serum FSH concentrations that precede ovarian follicular waves. These peaks can occur in the absence of follicular waves and even in the ovariectomized ewe (Barrett et al., 2006; Duggavathi et al., 2005a; Duggavathi et al., 2008) and do not appear to be regulated by changes in negative feedback by ovarian follicular secretory products such as estradiol and inhibin (Evans et al, 2001a; Souza et al., 1996; Souza et al., 1998; Souza et al., 1997).

In the present study, some FSH peaks were still noted 17 to 29 d after the cessation of pulsed LH secretion and ovarian follicular waves. It would appear that the regular peaks in serum concentrations of FSH that precede ovarian follicular waves in the ewe are not regulated by fluctuations in negative feedback by ovarian follicular secretory products from large antral follicles as they grow and regress. This regulation by ovarian hormones is seen in cattle and probably involves estradiol and inhibin (Driancourt, 2001; Ginther et al., 1996; Mihm and Evans, 2008). Interestingly, in cyclic ewes, serum concentrations of inhibin are not well correlated to the pattern of ovarian follicular waves and their associated peaks in serum FSH concentrations (Souza et al., 1998; Souza et al., 1997). In addition, in cyclic ewes, follicular waves were blocked but FSH peaks of reduced amplitude were seen in ewes given estradiol-releasing implants (Barrett et al., 2006). In anestrous ewes, the regular rhythm of FSH peaks and follicular

waves is not associated with any rhythm in the secretion of inhibin or estradiol (Bartlewski et al., 1998; Evans et al, 2001a; Souza et al., 1996). These findings are consistent with the existence of some endogenous rhythm that drives FSH peaks independent of changes in ovarian follicular feedback or acute changes in GnRH secretion (Duggavathi et al., 2005a). A separate FSH releasing hormone has been suggested (McCann et al., 1998; McCann et al., 1983; Padmanabhan and McNeilly, 2001; Padmanabhan and Sharma, 2001).

In the present study, in contrast to previous studies (Gonzalez-Bulnes et al., 2006; Lopez-Alonso et al., 2005), the number of small follicles was not affected in ewes immunized against GnRH. In the present study, the numbers of small follicles remained constant even when there was less gonadotropic support; however the lack of gonadotropic support inhibited their ability to progress into larger size categories. It is believed that the ability of FSH to stimulate the growth of multiple follicles is inhibited in the presence of high-frequency pulses of LH (Picton and McNeilly, 1991). Thus the residual FSH secretion that was seen in the present immunized ewes could have been sufficient to maintain the growth of small antral follicles in the absence of pulsed LH secretion. Maintenance of the small follicle pool would not appear to require regular secretion of LH pulses.

It is interesting that in the present study, serum estradiol concentrations were not affected by immunization. As in previous studies (Campbell et al., 1997; Campbell et al., 1998), we expected a decrease in estradiol concentrations as there was a decrease in serum LH concentrations and an absence of pulsatile LH secretion in immunized ewes after the booster immunization. Ewes are known to produce large follicles that are less

estrogenic during anestrous compared to the breeding season (Bartlewski et al., 1998). It was intriguing to see the gradual increase in estradiol concentrations from scanning period 1 to 3 within both control and ewes immunized against GnRH. These anestrous ewes progressed from mid anestrous to the time of transition to the breeding season (May to mid-July) during the study. Large ovarian follicles become more estrogenic in the transition from anestrous to the breeding season (Bartlewski et al., 1999c).

In summary, in anestrous ewes, when pulsed LH secretion was abolished by immunization against GnRH, the peaks in serum concentrations of FSH that trigger ovarian follicular waves continued for a period of time. Even at 17 to 29 d after the cessation of pulsed LH secretion and ovarian follicular waves, some FSH peaks were still noted. We concluded that although blocking the effects of GnRH gradually causes a diminution of FSH secretion, there is no acute requirement for GnRH in the regulation of FSH peaks. The existence of FSH peaks in the absence of follicular waves, and pulsed LH secretion, suggest that some endogenous rhythm may drive the occurrence of FSH peaks, independent of both changes in negative feedback by secretory products from ovarian antral follicles and GnRH.

# Chapter 7: MARKERS OF OVARIAN ANTRAL FOLLICULAR DEVELOPMENT IN SHEEP: COMPARISON OF FOLLICLES DESTINED TO OVULATE FROM THE FINAL OR PENULTIMATE FOLLICULAR WAVE OF THE ESTROUS CYCLE [*]

Seekallu SV, Toosi BM, Grazul-Bilska A and Rawlings NC

## 7.1 ABSRACT

Treatment of non-prolific Western White Face (WWF) ewes with prostaglandin F2alpha (PGF2a) and medroxy progesterone acetate (MPA) increased the ovulation rate as a result of ovulations from the penultimate wave in addition to the final wave of the cycle. The objective of the current study was to evaluate the expression of markers of vascularization/angiogenesis, a marker of intercellular communication, and cellular proliferation in follicles from the penultimate and final wave. On Day 8 of the estrous cycle, 15 ewes were given a single injection of PGF2a and an intravaginal MPA sponge, which remained in place for 6 d. Two days after the sponge removal, ovaries which contained follicles from the penultimate and final waves were collected and processed for immunohistochemistry followed by image analysis and quantitative real-time RT-PCR. Expression of factor VIII (marker of vascularization), proliferating cell nuclear antigen (PCNA) and connexin (Cx) 43 (marker of gap junctional communication) was greater in follicles from the final compared to the penultimate wave ($P<0.05$). For theca cells, mRNA expression for VEGF was greater ($P<0.05$) and for Cx43 and NOS3 tended to be greater ($P=0.1$ and $=0.05$) in follicles from the final wave compared to the penultimate wave. For granulosa cells, mRNA expression for Cx43 was greater ($P<0.05$) and for VEGF tended to be greater ($P=0.1$ and $=0.05$) in follicles from the final wave compared to the penultimate wave. In conclusion,

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extension of the lifespan of follicles in the penultimate wave seems to reduce follicular viability in the ewe.

## 7.2 INTRODUCTION

In cyclic ewes, ovarian antral follicles emerge and grow from a pool of small follicles (2 or 3 mm in diameter) in a wave like pattern, reaching diameters of =5 mm before regression or ovulation (Bartlewski et al., 1999a; Ginther et al., 1995; Schrick et al., 1993). In most cases ovulatory follicles develop from the cohort of follicles from the last follicular wave of the estrous cycle (Bartlewski et al., 1999a). However, ovulatory follicles can also be derived from the penultimate (second to last) wave of the cycle; which may ovulate at the same time as follicles from the last follicular wave of the cycle (Bartlewski et al., 1999a; Gibbons et al., 1999). The high ovulation rate in prolific Finn ewes is the result of ovulations from the penultimate wave in addition to the final wave of the cycle (Bartlewski et al., 1999b). Interestingly, serum progesterone concentrations in prolific Finn ewes are much lower compared to non-prolific Western White Face ewes (Bartlewski et al., 1999b).

In a previous study (Bartlewski et al., 2003), treatment of non-prolific WWF ewes with prostaglandin F2alpha (PGF2a) and medroxyprogesterone acetate (MAP) at midcycle changed follicular dynamics and increased ovulation rate by approximately 50% due to ovulations from both the penultimate wave and the final wave of the cycle. However, MAP and PGF2 alpha treatment in non prolific WWF ewes did not result in an increase in lambing rate (Davies, 2005). The ovulatory follicle from the penultimate wave is older compared to the ovulatory follicle from the final wave of the cycle.

However, the effect of the ovulation of aged follicles on fertility in sheep has not been investigated in detail (Evans, 2003a).

It has been demonstrated that follicular growth is regulated by numerous factors including angiogenic factors and extrafollicular and intrafollicular factors such as estradiol, insulin-like growth factors, insulin-like growth factor binding proteins and others (Echternkamp et al., 1994; Fortune et al., 2004; Hunter et al., 2005; Hunter et al., 2004). In fact, changes of vascularization and expression of some factors including angiogenic factors are associated with follicular growth and /or development or atresia (Fraser, 2006). Several angiogenic factors, including, vascular endothelial growth factor (VEGF), and endothelial nitric oxide synthase (NOS3), are expressed in ovarian follicles of several species (Grazul-Bilska et al., 2006; Redmer and Reynolds, 1996; Reynolds et al., 2002; Reynolds and Redmer, 1998).

Intercellular communication through gap junctions, which consist of a series of proteins called connexins (Cx), is also required for normal ovarian folliculogenesis (Grazul-Bilska et al., 1997b). Cx43 is the major gap junctional protein forming the channels within the granulosa cells and theca layers of the follicle (Kidder and Mhawi, 2002). Connexins play a significant role in the regulation of steroidogenesis, cell proliferation and also cell survival (Wiesen and Midgley, Jr., 1994). Therefore, cell proliferation and apoptosis, and Cx43 are also associated with follicular growth and development or atresia (17, 18, 40, 41, 45).

We hypothesized that vascularization, expression of several factors including Cx43, VEGF and NOS3, and cell proliferation will differ in ovarian follicles from the final compared to the penultimate wave of a cycle. We also hypothesized that the lack

of increased fertility in non-prolific WWF ewes, where ovulation was increased by PGF2a and MPA sponge treatment, could be partly explained by limited angiogenesis, limited cellular proliferation, and gap junction expression in preovulatory follicles from the penultimate wave compared to the final wave of a cycle. Therefore, the objective of the present study was to evaluate 1) the expression of mRNA for VEGF and NOS3, markers of angiogenesis; 2) the expression of factor VIII, a marker of endothelial cells and thus vascularization; 3) The labeling index, based on expression of proliferating cell nuclear antigen (PCNA), a marker of cellular proliferation; and 4) the expression of Cx43, a marker of gap-junctional communication, in preovulatory follicles obtained from the penultimate wave and final wave of the estrous cycle in WWF ewes treated with PGF2a and MAP.

## 7.3 MATERIALS AND METHODS

### 7.3.1 Animals, Ultrasound Technique, Blood Sampling

Care and handling of experimental animals was carried out according to the Canadian Council on Animal Care's published guidelines. Sexually mature, healthy, cycling, WWF ewes (n=15) were housed indoor for the duration of the study. The experiment was performed during the breeding season (December- January) and day length was set to ambient, outdoor conditions. Ewes were fed a maintenance diet of hay; cobalt iodized saltlicks and water was freely available. Ewes were monitored daily for estrus with vasectomized crayon marker-harnessed rams.

Ovarian antral follicular dynamics were monitored daily (starting at 0800 h) in all ewes by transrectal ovarian ultrasonography (scanning) using a 7.5-MHz linear transducer stiffened with a hollow plastic rod and connected to a B-mode, real-time

echo camera (Aloka SSD-900, Overseas Monitor, Richmond, BC, Canada). This technique can be used to accurately quantify ovarian antral follicles =2mm in diameter and detect corpora lutea in sheep (Duggavathi et al., 2003). All images were viewed at a magnification of X 1.5 with constant gain and focal point settings. Ovarian images were recorded (Panasonic AG 1978, Matsushita Electric, Mississauga, ON, Canada) on high-grade video tapes (Fuji S-VHS, ST-120 N, Fujifilm, Tokyo, Japan) for later examination. The relative position and dimension of follicles and luteal structures were also sketched on ovarian charts.

Blood samples (10 mL) were collected daily, after scanning, by jugular venipuncture, using vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA). All samples were permitted to clot at room temperature for 18 to 24 h. Samples were then centrifuged for 10 min at 1500 x g, and serum was removed and stored at !20 $^{o}$C until assayed for progesterone and FSH concentrations.

**7.3.2 Analysis of Follicular Data**

A follicular wave consisted of a follicle or a group of follicles that emerged and grew from 2 or 3 mm in diameter to =5 mm (growth phase), before regressing to 2 or 3 mm in diameter (regression phase) or ovulation; the period over which a follicle was maintained at a size of =5 mm was regarded as the static phase (Bartlewski et al., 1999a). The length of the growth and static phases, growth rate and life span of the largest follicle growing in the penultimate and final waves were analyzed. The lifespan of a large antral follicle was defined as the interval from its emergence at 2 or 3 mm to the day of follicle collection.

### 7.3.3 Experimental Design, Tissue Collection and Tissue Preparation

For all sheep, transrectal ovarian ultrasonography was performed to detect ovulation and continued from Day 8 after ovulation until overiectomy 8 d later to monitor ovarian antral follicular growth. Starting on Day 8 of the estrous cycle (Day 0 was the day of ovulation), all ewes were given a single injection of PGF2a (15 mg i.m.; Lutalyse, Upjohn, Orangeville, ON, Canada) and an intravaginal MPA sponge (60 mg; Veramix, Upjohn, ON, Canada), which remained in place for 6 d.

After sponge removal, we could clearly identify and monitor the development of large ovulatory follicles (=5 mm in diameter) from both the penultimate as well as from the final wave of the cycle. A map of ovarian follicular growth with time was created to allow us to identify the type of follicles we collected. Two days after the sponge removal, the ovaries which contained large preovulatory follicle(s) growing in the penultimate and final wave were collected by ovariectomy. Ovariectomy was performed by mid-ventral laparotomy within 5 min after euthanasia by Euthanyl Forte® (IV; 1 mL/5 kg of body weight; Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada). The ovaries were collected prior to ovulation which occurs approximately 72 h after the sponge removal (Bartlewski et al., 2003). Out of 15 ewes, ovaries from 8 ewes were used for gene expression studies and ovaries from 7 ewes were used for protein expression studies. All ewes had one follicle each from the final and penultimate waves of the cycle except for one ewe each in the gene expression and protein expression study groups which had two follicles from each of the final and penultimate waves, respectively.

Ovaries were collected and processed for mRNA and protein expression as previously described (Borowczyk et al., 2006; Grazul-Bilska et al., 2007). Briefly, ovaries were placed on ice and immediately transferred to the laboratory. Ovaries were washed using cold phosphate-buffered saline (PBS) solution containing 2% streptomycin and penicillin (Sigma, Oakville, ON), to remove blood from the ovarian surface. The ovaries were then placed on ice in a 60 mm petridish containing tissue culture medium-199 (TCM-199; Gibco, Grand Island, NY), supplemented with 1% penicillin and streptomycin.

For the protein expression studies, ovaries were collected and processed as previously described (Grazul-Bilska et al., 2007). Briefly, the ovaries were cut into small pieces which contained the identified follicle. A portion of a follicle was fixed in Carnoy's solution (6 parts of ethyl alcohol, 3 parts of chloroform and 1 part of glacial acetic acid) for 2-4 h, and another portion of follicle was fixed in 4% paraformaldehyde for 24 h at room temperature. Fixed tissues were dehydrated by using graded series of ethanol and cleared with a histological clearing agent (Histoclear®, National Diagnostics, Atlanta, GA). The tissues were embedded in paraffin and 4 µm sections were cut and mounted onto poly-L-Lysine coated slides for immunhistochemical staining.

For mRNA expression studies, granulosa and theca cells from the follicles were collected separately as previously described (Borowczyk et al., 2006). Follicular fluid from preovulatory follicles in the penultimate and final wave was aspirated and centrifuged to sediment the granulosa cells. The follicle was then flushed with TCM-199 media in order to maximize the yield of granulosa cells. The theca cell layer was

dissected from the follicle wall using fine tissue forceps. Granulosa cells and theca cells (all cell types from the theca layer) from the final and penultimate waves for each sheep were suspended in PBS and stored at -80 C until used for mRNA extraction.

### 7.3.4 Hormone Analysis

Progesterone (Rawlings et al., 1984) and FSH (Currie and Rawlings, 1989) concentrations were measured in serum samples by validated radioimmunoassay procedures. The assay sensitivities (defined as the lowest concentration of a hormone capable of significantly displacing radio-labeled hormone from the antibody) were: 0.03 ng/mL for progesterone and 0.1 ng/mL for FSH. The ranges of standards were: 0.1 to 5 ng/mL and 0.12 to 16.0 ng/mL, for the progesterone and FSH assays, respectively. A concentration equivalent to the sensitivity of the assay was assigned to serum samples with hormone concentrations lower than the assay sensitivity.

The intra- and inter-assay coefficients of variation (CVs) were 11.4% and 9.6% or 7.1% and 13.7% for reference sera with mean progesterone concentrations of 0.26 or 1.17 ng/mL, respectively. The intra-assay CVs were 2.2% or 4.0% for reference sera with mean FSH concentrations of 1.57 or 3.68 ng/mL, respectively.

Peaks of FSH in blood samples taken daily were identified using cycle-detection software (Clifton and Steiner, 1983). A fluctuation or cycle was defined as a progressive rise and fall in hormone concentrations that encapsulated a peak concentration (nadir-to-peak-to-nadir; Clifton and Steiner, 1983). Mean basal FSH concentrations were determined by averaging the lowest points between peaks (nadirs). Follicle stimulating hormone peak concentration was defined as the concentration of FSH observed at the apex of the FSH peak. Follicle stimulating hormone peak

amplitude was defined as the difference between the FSH peak concentration and the nadir before the peak concentration. Follicle stimulating hormone peak duration was defined as the interval between the two nadirs encompassing the FSH peak.

### 7.3.5 Immunohistochemistry

Detection of factor VIII, PCNA and Cx43, was performed as previously described (Grazul-Bilska et al., 2006; Grazul-Bilska et al., 2007; Pant et al., 2005; Vonnahme et al., 2008). Briefly, ovarian tissue sections were deparaffinized, rehydrated, and incubated with 3% $H_2O_2$ in methanol to eliminate endogenous peroxidase activity. The sections were then rinsed several times in PBS containing Triton X-100 (0.3%, vol/vol). Then, to block nonspecific binding of antibodies, sections were treated for 20 min with PBS containing either normal goat serum (1%, vol/vol; Vector labs, Burlingame, CA) for factor VIII and Cx43 staining or normal horse serum (3%, vol/vol; ABC kit, Vector Laboratories) for PCNA staining. The sections were incubated overnight at 4°C in PBS containing a primary antibody as follows: for factor VIII, rabbit polyclonal antibody raised against factor VIII (1:100 dilution; Sigma; (Grazul-Bilska et al., 2009)); for PCNA, monoclonal mouse antibody (1:500 dilution; Chemicon International, Temecula, CA;(Grazul-Bilska et al., 2007)) and for Cx43, rabbit polyclonal antibody raised against cx43 (a gift from Dr. W.J. Larsen, University of Cincinatti, Ohio, USA; (Pant et al., 2005)).

Primary antibodies were detected by using a biotin-labeled secondary antibody (anti-rabbit antibody for factor VIII and Cx43; anti-mouse antibody for PCNA; Vector Laboratories) and the Avidin Biotinylated Horse radish peroxidase macromolecular complex (Vector labs). The color was developed by using the SG substrate (Vector® SG

substrate kit, Vector laboratories) as described by (Grazul-Bilska et al., 2009; Grazul-Bilska et al., 2007).

For Cx43 and factor VIII controls, the primary antibody was replaced with normal rabbit IgG (diluted 1:100), and for PCNA controls, the primary antibody was replaced with normal mouse IgG (4 µg/mL). After immunostaining, the tissue sections were counter-stained with nuclear fast red to visualize the nuclei.

### 7.3.6 Image analysis

For all ovaries, images of stained sections (0.025 $mm^2$ per field) were taken for each of the identified follicles from the final and the penultimate wave (total 5-10 images/follicle). The images were then used for quantitative image analysis using the Image Pro-plus software (Media Cybernetics Inc., Silver Spring, MD). With this we determined the area that exhibited positive staining for factor VIII (occupied by endothelial cells) in the theca layer, or for Cx43 per tissue area, and the labelling index (LI; calculated as a percentage of proliferating cells out of the total cells per marked area of follicle) for granulosa and theca cells separately, as described previously (Grazul-Bilska et al., 2006; Vonnahme et al., 2006).

### 7.3.7 RNA Isolation and Quantitative Real Time RT-Polymerase Chain Reaction

Total cellular RNA was isolated from the granulosa and theca cells by using the illustra RNAspin Mini RNA Isolation Kit® (GE Healthcare UK limited, Amersham Place, Buckinghamshire, UK) according to the manufacturers recommendations. The quantity and quality of total cellular RNA samples were determined via capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California). The RNA samples were reverse transcribed in triplicate 20 µl reactions

using Taqman® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) as described previously by Johnson et al. (2007) and Redmer et al. (2005). All cDNAs from the reverse transcription reaction were stored at -20 °C prior to PCR analysis.

Expression of mRNA for VEGF, NOS3 and Cx43 in granulosa and theca cells were determined in the triplicate on the reverse transcribed (RT) samples described above using the ABI PRISM 7000® Sequence Detection system and software as described previously (Borowczyk et al., 2006; Redmer et al., 2005). For each gene assayed, a no-template control was present in duplicate on each real-time RT-PCR plate. The sequences of the primers and probes that were used for each gene studied have been published (Borowczyk et al., 2006; Johnson et al., 2006; Redmer et al., 2005). The primers and probes were designed from species-specific sheep mRNA sequences using the primer express software version 2 (Applied Biosystems). For standard curves, cDNA from placentomes (for VEGF and NOS3) at day 130 of pregnancy or from ovine heart (for Cx43) were used. As an RNA quantity control, 18S ribosomal RNA (rRNA) was used and it was analyzed using the 18S Pre-determined assay reagent (PDAR) kit from Applied Biosystems following the same PCR protocols. The concentrations of mRNA were then normalized to 18S rRNA by dividing each of the mRNA values by their corresponding 18S rRNA value (Johnson et al., 2006; Redmer et al., 2005; Vonnahme et al., 2006).

Ten micro-liter final reaction volumes were prepared in 96-well PCR plates fitted with optical adhesive covers (ABI prism™, Applied Biosystems) using the TaqMan™ universal PCR master mix (Applied Biosystems). Samples were heated to 60° C for 2 min, then 95°C for 10 min, before 40-45, 15 second cycles at 95° C; they

were finally heated at 60° C for 1 min as per the manufacturers instructions. The comparative $C_T$ method was used to analyze the Real-time data; $C_T$ is the cycle number at which the fluorescence reading is first recorded above background levels (Bustin, 2000). The $C_T$ method uses the arithmetic formula $2^{-??CT}$ to achieve relative quantification.

### 7.3.8 Statistical Analyses

One-way repeated measures ANOVA (Sigma Stat 7 for Windows Version 2.03, 1997, SPSS Inc.; Chicago, IL, USA) was used to assess differences in characteristics of FSH peaks (i.e., FSH peak concentrations and amplitude, FSH peak duration and basal FSH concentrations) amongst peaks during the experimental period studied. The t-test was used to compare the characteristics of ovarian follicles (i.e., length of growth and static phases, growth rate and life span) amongst the penultimate and final wave follicles. The t-test was also used to compare the relative mRNA expression and protein expression levels in granulosa and theca cells of preovulatory follicles in the final and penultimate waves. Data are expressed as mean ± S.E.M. Statistical significance was defined as $P<0.05$. A tendency for a statistical significance was defined as $P=0.05$ and $P=0.1$.

## 7.4 RESULTS

### 7.4.1 Mean Daily Serum Progesterone Concentrations

Approximately 24 h after PGF2a treatment on Day 8 of the estrous cycle, serum progesterone concentrations dropped ($P<0.05$) to less than 1 ng/ml, and remained at low level until collection of ovaries (Figure 7.1).

### 7.4.2 Antral Follicle Development

After PGF2a and MPA treatment preovulatory follicles emerged from both the final and penultimate wave of the cycle (Figure 7.2).The mean days of wave emergence were 8.9 ± 0.2 and 13.0 ± 0.2 after ovulation for penultimate and final waves, respectively (Figure 7.2). Preovulatory follicles from the penultimate wave had a longer ($P<0.05$) lifespan compared to the follicles from the final wave (7.1 ± 0.2 vs 3.0 ± 0.2 d; $P<0.05$; Figure 7.2). The static phase of preovulatory follicles from the penultimate wave was longer ($P<0.05$) compared to the follicles from the final wave (3.6 ± 0.7 vs 0.4 ± 0.2 d; Figure 7.2). However, the growth phase (3.7 ± 0.6 vs 2.6 ± 0.9 d) and the growth rate (0.9 ± 0.2 vs 1.1 ± 0.1 mm/day) did not differ amongst the follicles from the penultimate and the final waves (Figure 7.2). The mean maximum follicle diameter at the time of follicle collection did not differ among the preovulatory follicles from the penultimate (5.4 ± 0.2 mm) and final (5.55 ± 0.2 mm) waves (Figure 7.2).

### 7.4.3 Characteristics of Serum FSH concentrations

The mean day of the estrous cycle when the FSH peaks triggered the penultimate and final waves were 8.9 ± 0.3 and 12.7 ± 0.3, respectively (Figure 7.2). There were no differences in FSH peak concentration, amplitude and duration, or basal FSH concentration among the peaks that triggered the penultimate and final waves (Figure 7.2).

### 7.4.4 Immunohistochemical localization and image analysis

Factor VIII, PCNA and Cx43 were detected in follicles from the final wave and the penultimate wave (Figure 7.3). Factor VIII was immunolocalized to blood vessels in

the theca layer (Figure 7.3A, 7.3B), but PCNA (Figure 7.3C, 7.3D) and Cx43 (Figure 7.3E, 7.3F) were detected in granulosa and theca layer of ovarian follicles.

Expression of factor VIII was greater ($P<0.05$) in the theca layer of preovulatory follicles from the final compared to the penultimate wave (Figure 7.4A). The labeling index in granulosa cells was greater ($P<0.05$) and in theca cells tended ($P=0.6$) to be greater, in preovulatory follicles from the final wave compared to the penultimate wave (Figure 7.4B). Cx43 expression was greater ($P<0.05$) in both granulosa and theca cells of preovulatory follicles from the final wave compared to the penultimate wave (Figure 7.4C).

### 7.4.5 Relative mRNA expression

Expression of VEGF mRNA was greater ($P<0.05$) in theca cells and tended ($P<0.06$) to be greater in granulosa cells, of preovulatory follicles from the final wave compared to follicles from the penultimate wave (Figure 7.5). Expression of NOS3 mRNA tended ($P=0.06$) to be greater in theca cells of preovulatory follicles from the final wave compared to follicles from the penultimate wave (Figure 7.5, bottom panel). Expression of NOS3 mRNA in granulosa cells was similar ($P>0.1$) in the follicles from the final and penultimate waves (Figure 7.5, top panel). Expression of Cx43 mRNA was greater ($P<0.05$) in granulosa cells and tended ($P=0.09$) to be greater in theca cells of preovulatory follicles from the final wave compared to follicles from the penultimate wave (Figure 7.5).

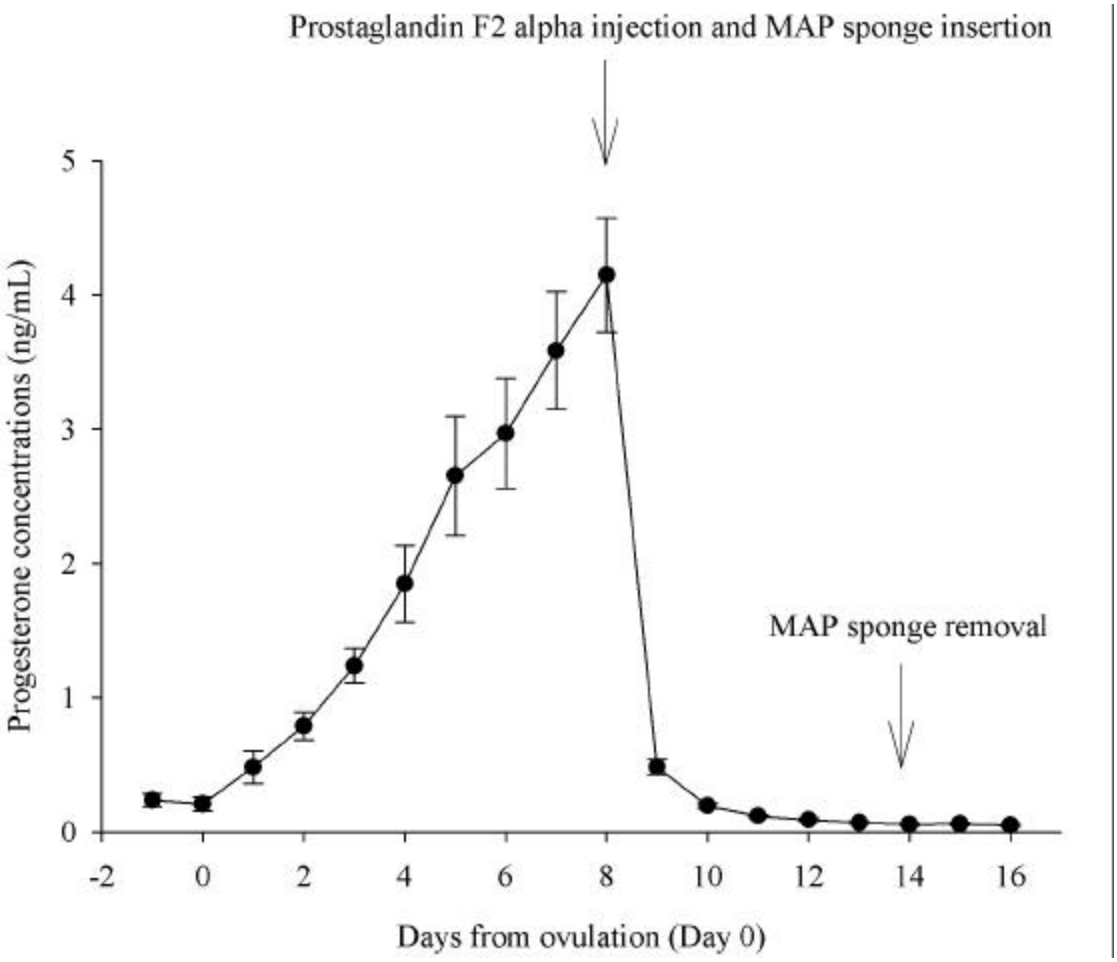


Fig. 7.1. Daily serum concentrations of progesterone (mean ± S.E.M) throughout the experimental period in fifteen cyclic Western White Face ewes that received PGF2a injection on Day 8 and had MAP intravaginal sponges in place from Days 8 to 14 after ovulation. Data is normalized to the day of ovulation (Day 0).

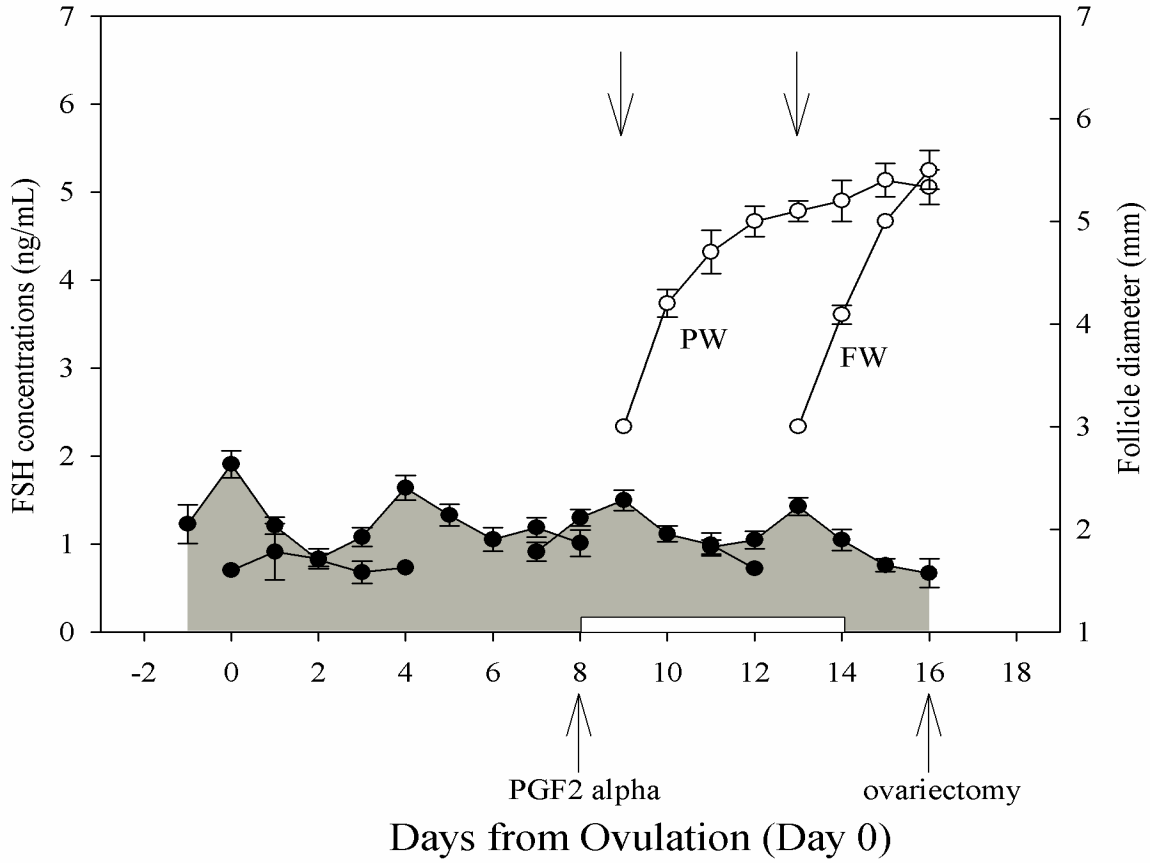


Fig. 7.2. Peaks in serum concentrations of FSH (outlined with shading) and their associated emerging follicular waves in fifteen cyclic Western White Face ewes that received PGF2a injection on Day 8 and MAP sponge from Days 8 to 14 (indicated by the rectangle box on x-axis) after ovulation. Data were normalized to the day of ovulation (Day 0) in all ewes. The average curves representing the growth, static and regression phases of all ewes in a group were normalized for each follicle wave to the mean day of wave emergence (indicated by the arrows; (Barrett et al., 2006)). All FSH peaks for all ewes are shown normalized to the mean day of occurrence of the apex of the FSH peak for each wave. Please note that transrectal ultrasonography was done initially to detect ovulation at the beginning of the study and from Day 8 until ovariectomy to monitor antral follicular development. Therefore, we could only detect the growth patterns of the second last [penultimate wave (FW)] and last [final wave (FW)] wave. For every FSH peak, serum concentration profiles were delimited by the encompassing nadirs of the FSH concentrations (hence the overlap of the data for adjacent peaks in some cases).

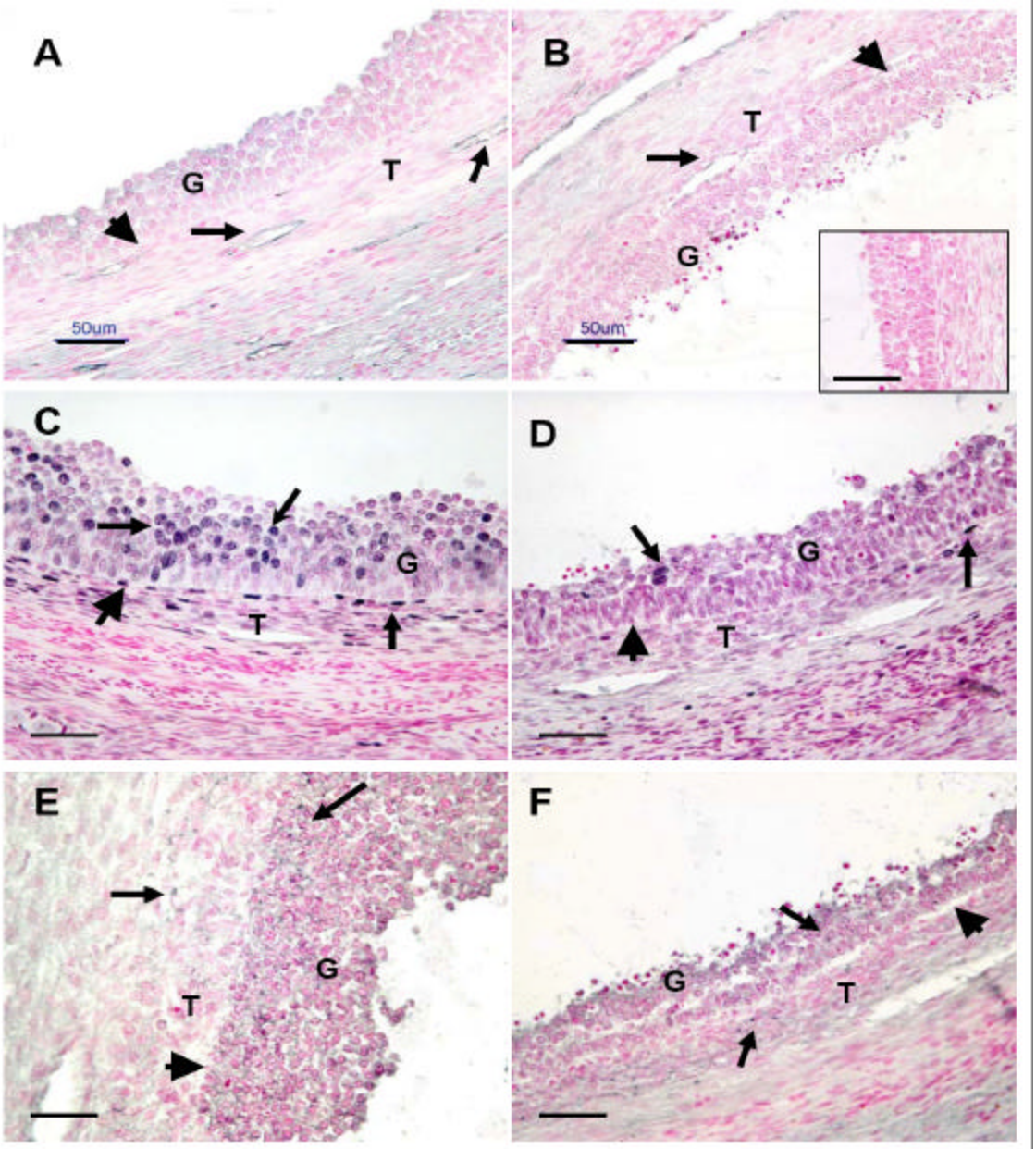


Fig. 7.3. Representative micrographs of immunohistochemical staining for factor VIII (A,B), PCNA (C,D) and Cx43 (E,F) in preovulatory follicles from final (left column) and penultimate (right column) wave obtained from WWF ewes. Ewes received $PGF_{2a}$. Injection on Day 8 and had MPA intravaginal sponges in place from days 8 to 14 after ovulation. The dark color (bluish grey or blackish) indicates positive staining (arrows), and pink staining indicates cell nuclei (fast red staining) in granulosa (G) and theca (T) layers. The large white area is the follicular antrum. Negative controls (insert in B) did not exhibit any staining. Arrowheads identify the basement membrane. Note the relatively stronger and weaker positive staining in final and penultimate wave preovulatory follicles, respectively. Size of the bar = 50 µm.

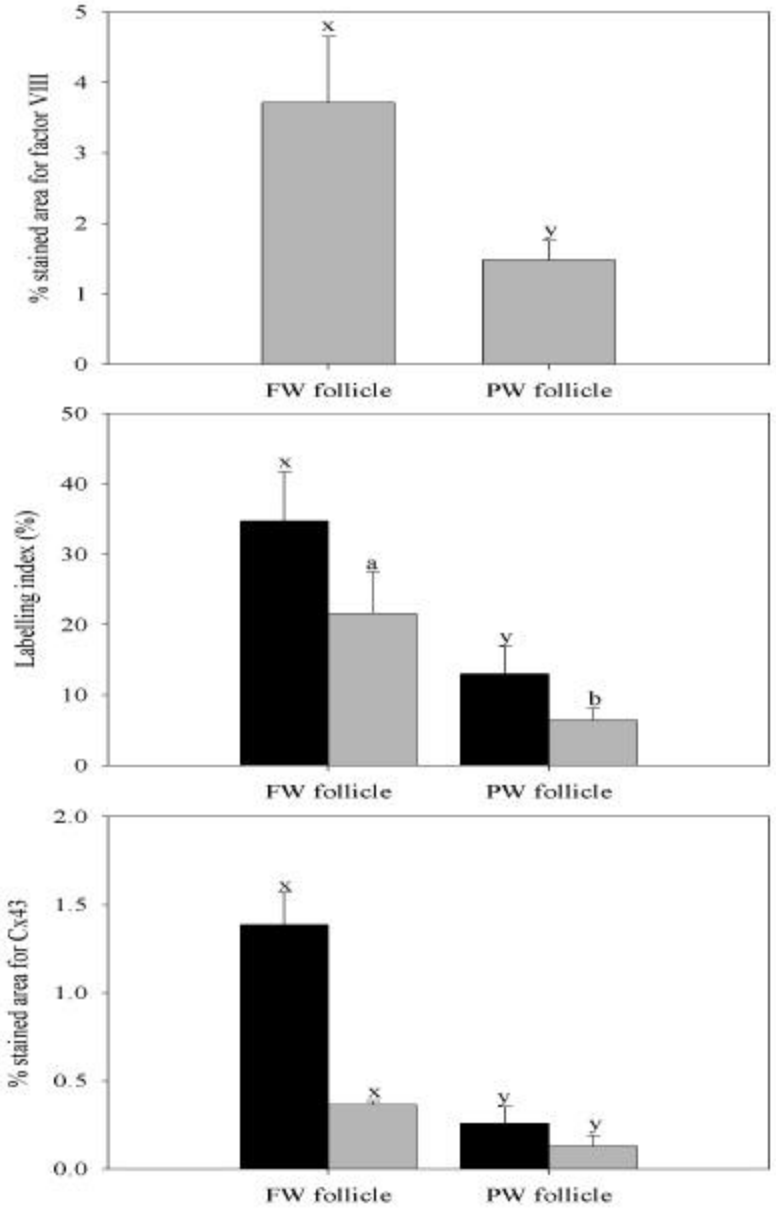


Fig. 7.4. Factor VIII (top panel), Labeling index (as measured by PCNA; middle panel), and expression of Cx43 (bottom panel) in granulosa (black bars) and theca cells (grey bars) of final and penultimate wave preovulatory follicles obtained from seven cyclic Western White Face ewes. Ewes received PGF2a injection on Day 8 and had MAP intravaginal sponges in place from Days 8 to 14 after ovulation.

[x,y] $P<0.05$, values with different superscripts show differences between the final and penultimate wave follicles.

[a,b] $P \geq 0.05$ but $=0.1$, values with different superscripts show a tendency to differ between the final and penultimate wave follicles.

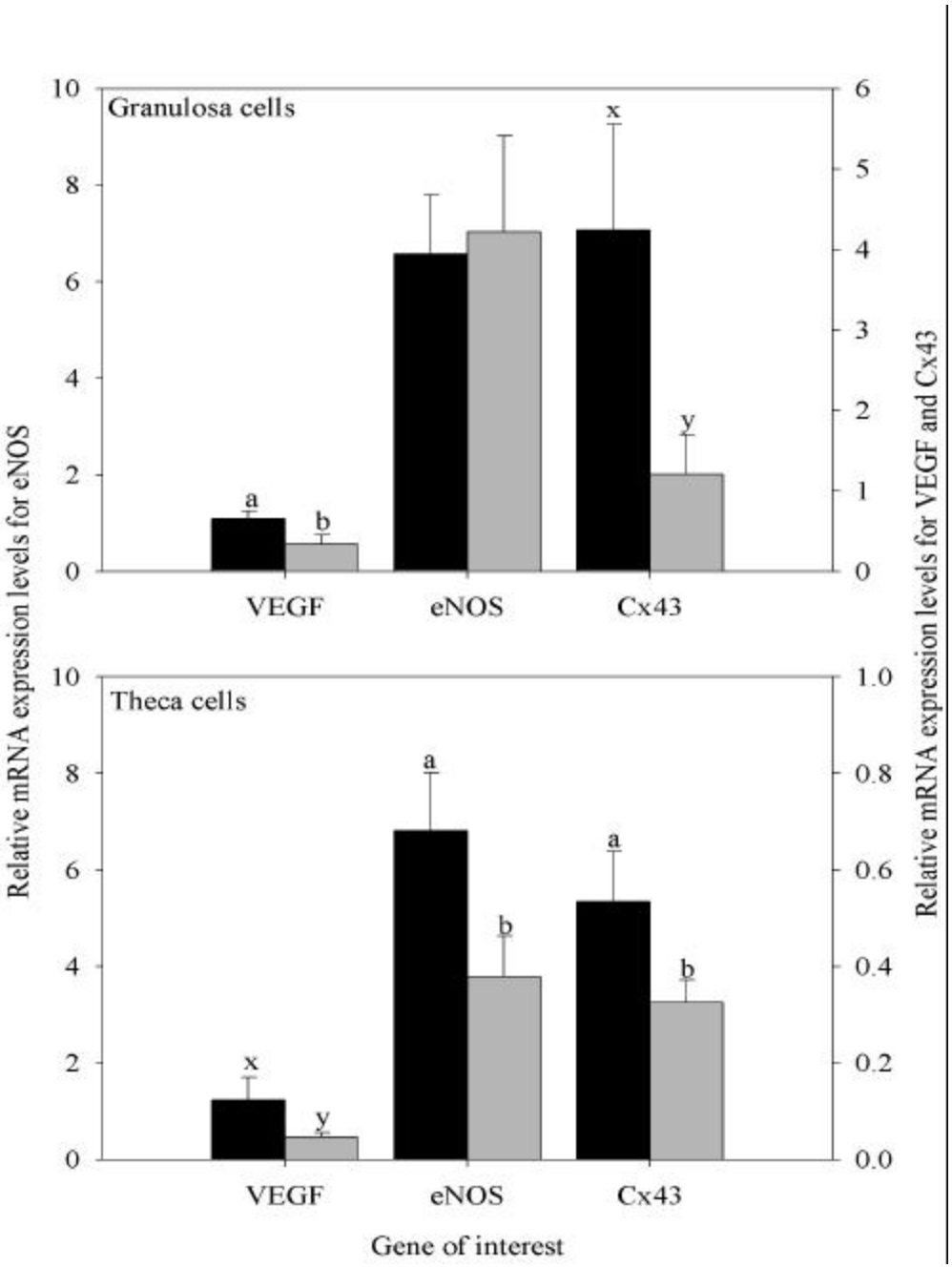


Fig. 7.5. Relative mRNA expression for VEGF, NOS3 and Cx43 in granulosa (top panel) and theca cells (bottom panel) of final (black bars) and penultimate wave (grey bars) preovulatory follicles obtained from eight cyclic Western White Face ewes. Ewes received PGF2a injection on Day 8 and had MPA intravaginal sponges in place from Days 8 to 14 after ovulation.

[x,y] $P<0.05$, values with different superscripts differ for the final and penultimate wave follicles.

[a,b] $P\geq0.05$ but $=0.1$, values with different superscripts tend to differ for the final and penultimate wave follicles.

## 7.5 DISCUSSION

In agreement with previous studies (Bartlewski et al., 2003; Johnson et al., 1996; Leyva et al., 1998; Vinoles et al., 1999), PGF2a and MPA treatment resulted in the development of preovulatory follicles from the penultimate wave in addition to the final wave of the cycle. The preovulatory follicles from the penultimate wave had a longer lifespan compared to the final wave. The extended follicular lifespan of the follicles from the penultimate wave may not depend on changes in gonadotropin secretion (Bartlewski et al., 2003). As presented in the present study, there was no difference among any of the characteristics of the FSH peaks that triggered either the penultimate or the final wave of the cycle. It has been suggested that the low serum concentrations of progestagen from MAP sponges exert direct effects on the ovary and affect follicular function (Bartlewski et al., 2003). However, it is interesting that follicles could grow for different lengths of time, to the same size, with similar FSH peak characteristics; this phenomenon warrants further investigation.

In the present study, overall, the expression of markers of vascularization and/or angiogenesis such as VEGF, NOS3 and factor VIII was greater in preovulatory follicles from the final wave compared to penultimate wave, even though follicle size was similar. The mRNA expression for NOS3, VEGF and bFGF was detected in both theca and granulosa cell layers of the follicles (Grazul-Bilska et al., 2006). It has been shown for sheep and cattle, that the expression of angiogenic factors such as VEGF and NOS3 is associated with enhanced vascularization of large ovarian antral follicles, which likely contributes to maintaining these follicles in a non-atretic state (Grazul-Bilska et al., 2006; Grazul-Bilska et al., 2007; Redmer and Reynolds, 1996). Both VEGF and

NOS3 are major regulators of follicular development in sheep (Fraser, 2006; Grazul-Bilska et al., 2006; Hunter et al., 2004). The high expression of angiogenic factors and increased vascular surface area, as indicated by factor VIII expression, likely result in enhanced vascular supply and hence enhanced exchange of nutrients, gonadotropins and growth factors in follicles in the final compared to the penultimate wave of the cycle, in the present study (Grazul-Bilska et al., 2006; Grazul-Bilska et al., 2007). In fact, it has been demonstrated that vascularity in the theca layer may serve as an indicator of follicle health in several species (Jiang et al., 2003; Mattioli et al., 2001). Even though, PGF2a and MPA treatment resulted in large ovulatory sized follicles in both the final and penultimate waves of the estrous cycle, the quality of the follicles in the penultimate wave was compromised with respect to vascularization and angiogenesis compared to the final wave.

In the present study, proliferative activity of granulosa and theca cells, as detected by expression of PCNA, was greater in follicles in the final compared to the penultimate wave of the cycle. In cattle, it was shown that cellular proliferation in granulosa and theca layers was greater in estrogen-active and dominant follicles than in estrogen inactive and non-dominant follicles (Grazul-Bilska et al., 2007). In addition, a greater proliferation rate was observed in healthy compared to atretic follicles in sheep and cattle (Isobe and Yoshimura, 2000; Jablonka-Shariff et al., 1994). Thus, these data demonstrate that in follicles from the penultimate wave, with an extended static phase, there is a decrease in granulosa and theca cell proliferation leading to follicle atresia and likely poor oocyte quality.

Both protein and mRNA expression for Cx43 was greater in final wave compared to penultimate wave follicles in the present study. The importance of Cx43, the major gap junctional protein in ovaries, during follicular development has been well recognized (Gershon et al., 2008; Grazul-Bilska et al., 1997b; Kidder and Mhawi, 2002). Gap junctions are required for granulosa and theca cell proliferation, steroidogenesis, signal transduction and cell survival (Grazul-Bilska et al., 1997b; Kidder and Mhawi, 2002). Based on studies in knockout mice, ovaries lacking Cx43 have decreased intracellular coupling between granulosa cells leading to decreased follicular growth and increased apoptosis of granulosa cells, and a lack of ovulation (Ackert et al., 2001; Gittens et al., 2005). In addition, it has been shown that the level of Cx43 mRNA expression in human preovulatory follicles or the bovine cumulus oocyte complex, predicts oocyte developmental competence (Calder et al., 2003; Tsai et al., 2003). Moreover, decreased expression of Cx43 was observed in atretic follicles in sheep and cattle (Grazul-Bilska et al., 1998; Johnson et al., 1999). Therefore, Cx43 appears to be critical for follicular development and thus can be used as a marker for follicle quality. In the present study, due to low expression of Cx43, gap junctional communication was likely limited within granulosa and theca cells of follicles in the penultimate compared to the final wave of a cycle, probably compromising oocyte quality.

One of the primary goals in controlled sheep breeding is to increase the number of live births from a ewe. A field study, utilizing the treatment used in the present study did not improve the fertility rate, even though there was an increased ovulation rate (Davies, 2005). The findings of the present study clearly show that the inability of the

PGF2a and MPA treatment to increase fertility could be partly due to a decrease in the quality of preovulatory follicles in the penultimate wave of the estrous cycle, whose lifespan was extended (Bartlewski et al., 2003). In cattle, oocyte quality is compromised in older follicles and fertility is decreased (Austin et al., 1999; Mihm et al., 1999). In contrast, (Evans et al., 2001b) demonstrated that oocytes from follicles with a prolonged lifespan could be fertilized and produce good quality embryos in sheep. These discrepancies are likely due to different hormonal treatment and/or species. Therefore, additional hormonal treatments should be developed to enhance follicular development from the penultimate wave of a cycle, to enhance fertility and/or fecundity.

In summary, PGF2a and MPA treatment gave rise to preovulatory follicles emerging both from the final and penultimate wave of the cycle in WWF ewes. Follicles from the penultimate wave had a longer lifespan and static phase compared to follicles from the final wave. Vascularization, cell proliferation, and expression of angiogenic factors and gap junctional protein Cx43 were greater in preovulatory follicles from the final wave compared to follicles from the penultimate wave. This indicates that although the treatment with PGF2a and MPA enhanced ovulation rate by extending the lifespan of follicles in the penultimate wave, these follicles were not as functionally viable as follicles in the final wave. Moreover, factors determined in the present study (e.g., angiogenic factors, Cx43, call proliferation and apoptosis) could be used as markers of follicle health and atresia. Furthermore, PGF2a and MPA treatment provides a useful model to study aged antral follicles and oocytes, but this treatment is not useful for enhancing fecundity.

# Chapter 8: GENERAL DISCUSSION, CONCLUSIONS, PRACTICAL IMPLICATIONS AND FUTURE STUDIES

## 8.1 GENERAL DISCUSSION

The first study in this thesis was designed to examine the characteristics of follicular waves and hormonal profiles in ewes with three or four follicular waves in an estrous cycle. The role of pulsatile LH secretion in the emergence and growth of follicular waves was also examined; as was the role of GnRH in regulating FSH secretory peaks. Further, the effect of follicular lifespan on markers of follicular viability was also assessed.

### 8.1.1 Regulation of the number of follicular waves in an estrous cycle

Two to four follicular waves per estrous cycle have been reported in different breeds of sheep (Bartlewski et al., 1999a; Contreras-Solis et al., 2008; Evans et al., 2000; Ginther et al., 1995; Noel et al., 1993; Ravindra et al., 1994). The length of the estrous cycle is consistent among different breeds and is not affected by age (McKinszie and Terrill, 1937). Interestingly, we found that the length of the estrous cycle did not differ between cycles with three or four waves (chapter 2). This finding is novel and has not been reported previously. In fact, to our knowledge, there have been no other studies looking at the differences between cycles with different numbers of follicular waves in sheep. In contrast, there are reports in cattle, looking at the characteristics of cycles with two or three waves (Celik et al., 2005; Ginther et al., 1989; Jaiswal et al., 2009; Noseir, 2003). In cattle, the length of the estrous cycle was extended by a few days, when the number of follicular waves was increased from two to three per cycle

(Celik et al., 2005; Ginther et al., 1989; Noseir, 2003). However, in another study (Savio et al., 1988) the length of the estrous cycle appeared to be the same in cycles with two or three waves.

In the present studies in sheep, in moving from three to four waves per cycle, the inter-wave intervals at the beginning and end of the cycle were shortened; but no consistent trends or changes in the length of specific phases of a wave were noted that explained this. There were no apparent endocrine or follicular characteristics that enabled us to develop a mechanism to explain how three or four waves could be incorporated into a cycle without altering the length of the cycle. In fact, the clearest trends were for endocrine and follicular characteristics to change across the cycle in a similar manner regardless of the number of waves per cycle. Comparing inhibin concentrations in serum between cycles with three or four waves would be interesting. In cattle, it was shown that inhibin concentrations reached their peak in plasma sooner during the first wave of a cycle containing two compared to three waves (Parker et al., 2003). *In this thesis work, it was not possible to ascertain clearly any particular factor regulating the number of waves per cycle in the ewe.* Using a greater number of sheep and by examining other factors such as inhibin, may be useful approaches to answering this question.

### 8.1.2 FSH and follicular waves in the ewe

FSH peaks continued to occur in the absence of follicular wave development in anestrous ewes (chapters 3, 5, and 6). It appears that peaks in serum FSH secretion occur independent of negative feedback effects of secretory products secreted from the large follicles growing in a wave (chapters 3, 5, and 6). In contrast, in cattle it has been

shown that peaks in FSH secretion are governed by secretory products from follicular waves; such as estradiol and inhibin (Beg et al., 2002; Mihm et al., 2006; Mihm and Evans, 2008). In sheep, peaks in serum FSH concentrations and the associated follicular waves, are not regulated by estradiol and inhibin in anestrous ewes as serum concentrations of estradiol and inhibin do not change with the pattern of follicular waves in the non-cycling ewe (Evans et al, 2001a). In a previous study in cyclic ewes, it was shown that estradiol releasing implants blocked emergence of follicular waves, however, FSH peaks of low amplitude continued to occur at the expected frequency (Barrett et al., 2006). FSH peaks are also seen in ovariectomized ewes with a rhythm similar to that seen in the intact ewe (Duggavathi et al., 2005a).

FSH secretion from the anterior pituitary is comprised mainly of constitutive basal secretion with a minor pulsatile component (McNeilly, 1988; Padmanabhan and Sharma, 2001). Pulses of FSH secretion are seen in the peripheral circulation in ovariectomized ewes, but only in the hypophyseal-hypothalamic portal system in intact ewes (Padmanabhan et al., 2003; Padmanabhan et al., 1997; Padmanabhan and Sharma, 2001; Wallace and McNeilly, 1986). In ewes, where the effects of GnRH were blocked by use of GnRH immunization (Clarke et al., 1998; Mariana et al., 1998) or GnRH agonists (McNeilly and Fraser, 1987) and antagonists (Campbell et al., 1997; Campbell et al., 1998; Padmanabhan et al., 2003), it was demonstrated that pulsatile secretion of LH was abolished immediately; while, mean serum FSH secretion was only partially suppressed or not altered. The fate of FSH peaks in these latter studies was; however, not investigated. In chapter 6 of this thesis, it was shown that peaks in serum FSH secretion were present even after the effects of GnRH were blocked by immunization.

We could conclude that FSH peaks are not acutely dependent on pulsed GnRH secretion. *Results from the present thesis and from the work of others, would support the concept of a GnRH independent endogenous rhythm that drives peaks in serum FSH secretion in sheep.* A separate FSH releasing factor has been suggested (McCann et al., 1998; McCann et al., 1983; Padmanabhan and McNeilly, 2001; Padmanabhan and Sharma, 2001).

In chapter 4 of this thesis, we proposed the existence of "induced follicular dominance" in sheep. In this study, one group of ewes was treated with frequent injections of GnRH to create an LH pulse frequency, typical of the follicular phase, but in the mid-luteal phase of the cycle. Treatment appeared to block an FSH peak. Obviously, this resulted in the loss of a follicular wave. We assumed that the increased serum concentrations of estradiol and progesterone caused by GnRH treatment suppressed FSH secretion and blocked the peak in serum concentrations of FSH. Some form of limited dominance in sheep has been suggested by some researchers (Evans, 2003b; Gonzalez-Bulnes et al., 2001; Gonzalez-Bulnes et al., 2004; Gonzalez-Bulnes and Veiga-Lopez, 2008). In several earlier studies, the creation of supraphysiological serum concentrations of estradiol and progesterone also suppressed FSH peaks (Barrett et al., 2006; Barrett et al., 2007; Rawlings et al., 1984). Nevertheless, it has been demonstrated that physiological peaks in serum concentrations of FSH could be created in cyclic ewes, during the inter-wave interval, by injections of oFSH. Follicular waves could be induced even if the oFSH was given during the growth phase of a follicular wave resulting from an endogenously generated FSH peak (Duggavathi et al., 2004). The large growing antral follicles of each wave in sheep do not appear to suppress the

growth of small follicles as seen in cattle (Driancourt et al., 1991; Duggavathi et al., 2004). *Therefore, more and more evidence is accumulating to question the existence of follicular dominance in sheep.*

It is a well established fact that peaks in serum FSH concentrations precede the emergence of follicular waves in both cyclic and anestrous ewes and are required to trigger the emergence of follicular waves (Bartlewski et al., 1999a; Bartlewski et al., 1998; Bister et al., 1999; Duggavathi et al., 2004; Duggavathi et al., 2003; Ginther et al., 1995; Souza et al., 1998). The role of FSH peak concentration and amplitude and basal FSH secretion in the development of follicular waves is still unclear. Based on the results presented in chapter 2 of this thesis, it appears that there is some kind of temporal association between FSH peak concentration and amplitude with that of the length of the life span and regression phase of the largest follicle growing in waves. The length of the lifespan and the regression phase declined across the cycle as FSH peak concentration and amplitude decreased.

However, based on the results from studies presented in chapter 7 of this thesis, it appeared that there were no differences in either concentrations and/or amplitude of the FSH peaks that triggered the growth of the penultimate and final waves of a cycle. The largest follicle growing in the penultimate wave had a longer lifespan compared to that of the final wave even though the preceding FSH peaks were of similar concentration and amplitude. However, some specific mechanism may exist to prolong the lifespan of follicles in a penultimate wave to allow them to ovulate independent of the FSH peak that precedes the wave. Therefore, although *FSH peaks trigger follicular*

*waves, the amplitude of these peaks may not affect characteristics of the growth of follicular waves.*

Treatment of cyclic ewes with implants releasing estradiol and progesterone decreased the amplitude of FSH peaks, blocked follicular waves and reduced the pool of small follicles (= 1 mm to =3 mm in diameter) in the ovary (Barrett et al., 2007). It was concluded from this study that FSH peaks are required to maintain the pool of small follicles in the ovary of cyclic ewes (Barrett et al., 2007). In contrast, in anestrous ewes, the creation of supra-physiological concentrations of estradiol and physiological concentrations of progesterone together decreased FSH peak amplitude, blocked follicular waves but resulted in an increase in the number of follicles in the small follicle pool (chapter 5). *The effect of FSH peak amplitude in maintenance of the small follicle pool in the ewe is not clear and obviously needs further investigation.*

Implants releasing only estradiol in anestrous ewes (chapters 3 and 5) had no effects on the number of follicles in the pool of small follicles. In these ewes, pulsed LH secretion was abolished but only limited effects were seen on FSH secretion. Interestingly, in chapter 6 of this thesis, in contrast to previous studies (Gonzalez-Bulnes et al., 2006; Lopez-Alonso et al., 2005), the number of small follicles were not affected in ewes immunized against GnRH. In these ewes, pulsed LH secretion was completely abolished as soon as appreciable antibody titres to GnRH were seen; however, FSH secretion was still present for some time. Thus the remaining FSH secretion, in the immunized ewes, could have been sufficient to maintain the growth of small antral follicles in the absence of pulsed LH secretion. Earlier studies on the endocrine regulation of follicular development in sheep have shown that ovarian antral

follicles, 2-3 mm in diameter and larger are dependent on gonadotropins. This dependence is largely for FSH but basal secretion of LH may also be required (Campbell et al., 1998; McNeilly et al., 1982; McNeilly et al., 1991b; Picton et al., 1990a). Serum concentrations of FSH drop significantly after luteal regression and prior to ovulation, due to an increase in secretion of estradiol and inhibin by ovulatory follicles (Baird, 1983; Baird and McNeilly, 1981). Therefore, it is believed that final maturation and development of ovarian antral follicles to ovulation is dependent on an increase in pulsatile secretion of LH (Baird, 1983; Baird and McNeilly, 1981; Hillier, 2001). *Based on the observations above, it appears that maintenance of the pool of small follicles in the ovine ovary does not require regular pulsed secretion of LH but that this pool of follicles can be maintained by FSH and perhaps basal serum concentrations of LH.*

### 8.1.3 Regulation of follicular waves by LH

LH is secreted from the anterior pituitary in a pulsatile manner. The frequency of pulses is lower during the luteal phase, when serum progesterone concentrations are high, compared to other phases of the estrous cycle, when serum progesterone concentrations are low (Leyva et al., 1998). The frequency of LH pulses is regulated by progesterone which is mainly secreted from the corpus luteum (Martin et al., 1988; Rawlings et al., 1984; Thiery and Martin, 1991). The role of pulsatile LH secretion in the emergence and growth of ovarian antral follicles is unclear. Steroid-releasing implants have been used, to differentially regulate LH and FSH secretion to study their respective roles in the development of antral follicular waves in sheep (Barrett et al., 2006; Barrett et al., 2007). In chapter 2 of this thesis, fewer follicles entered Wave 1 and

the last or ovulatory wave compared to other waves in the cycle but the largest follicles growing in the first and last wave had a greater maximum follicle diameter compared to follicles growing in other waves. We assumed that the follicles in the Wave 1 and the ovulatory wave grew larger as these follicles were exposed to a greater frequency of LH pulses compared to follicles growing in the luteal phase of the cycle (Baird, 1978; Martin, 1984; Rawlings and Cook, 1993; Wheaton et al., 1984). It was previously demonstrated that follicles were larger and grew for a longer period of time when ewes were treated with serum progesterone concentrations that were lower than those seen in the luteal phase of a cycle (Vinoles et al., 2001). In the present study, in general, the numbers of follicles 4 mm and = 5 mm in diameter were lowest during the first and last/ ovulatory waves of the cycle (chapter 2); again, when LH pulse frequency was greatest. *Based on the findings above, we concluded that for the first and the last waves of the cycle in the ewe, when the ovary is exposed to a high frequency of LH pulses, fewer follicles enter follicular waves but these follicles grow to a larger size than those seen in other waves of the cycle.*

In the present studies, in anestrous ewes, one of the most profound effects of implants releasing steroids was on follicle development (chapters 3 and 5). In anestrous ewes given implants releasing estradiol-17ß alone, pulsatile LH secretion was abolished and follicular wave emergence was blocked, with only minimal effect on the amplitude, or number of peaks in serum FSH concentrations (chapters 3, 5). Alternatively, immunization of anestrous ewes against GnRH (chapter 6), resulted in the loss of follicular waves. Pulsed LH secretion was immediately abolished following immunization but peaks in serum FSH secretion remained for some time. We assumed

that in these studies above, because LH pulses were suppressed, follicles failed to receive the appropriate LH support and had difficulty growing beyond 2 mm in diameter, even though FSH peaks were seen in these anestrous ewes. In fact, in chapter 3 of this thesis, in anestrous ewes, where pulses in serum concentrations of LH were suppressed by treatment with implants releasing estradiol, LH pulses were restored by GnRH injections and follicular waves were re-established. We should note that GnRH injections did transiently increase serum FSH concentrations for up to 1 hr. However, these increases did not cause changes in the peaks in serum FSH concentrations that precede ovarian follicular waves, in comparison to control ewes (chapter 3). *Based on these findings, we concluded that some level of pulsatile LH secretion is required for the peaks in FSH secretion to induce follicular waves in the anestrous ewe.*

The temporal association between the secretory patterns of LH and characteristics of follicular waves have been studied, both during the period from metestrus to the early luteal phase and during the mid to late luteal period of the estrous cycle (Bartlewski et al., 2000a; Duggavathi et al., 2005b). From the latter studies, it was concluded that there are no consistent and major regulatory interactions between the secretory patterns of LH and follicular waves, rather there was a correlation of LH secretory patterns with growth and regression of the corpus luteum (Bartlewski et al., 2000a; Duggavathi et al., 2005b). Ewes in these two studies would have experienced frequencies of pulsed LH secretion ranging from the low frequencies of the mid to late luteal phase to the higher frequencies of the early luteal phase of the cycle (Bartlewski et al., 2000a; Duggavathi et al., 2005b). In another recent study (Campbell et al., 2007), using ewes with ovarian transplants and given a GnRH antagonist, it was demonstrated

that antral follicles grew in the same way irrespective of whether treatment with LH was given in the form of constant infusion or pulses. However, the change in size of the follicles was not significant and was not enhanced by treatment with either the pulses of LH or the constant infusion of a low dose of LH (Campbell et al., 2007). Increasing the dose of LH given by constant infusion did result in a significant increase in follicle size (Campbell et al., 2007). In the present studies, when cyclic ewes were exposed to an increased LH pulse frequency induced by treatment with GnRH, during the luteal phase of the cycle, large antral follicles in a follicular wave grew to a greater size than the equivalent follicles in the control ewes (chapter 4). Interestingly, the follicles above grew and functioned in terms of estradiol production, like ovulatory follicles growing at the end of a cycle, even though they were induced to grow in a mileu of high serum concentrations of progesterone (chapter 4). It is intriguing that a follicular wave equivalent to an ovulatory wave can be induced in the luteal phase of the cycle by increasing LH pulse frequency to that seen during the follicular phase (chapter 4). The presence of serum concentrations of progesterone typical of the luteal phase did not directly inhibit a follicle from growing to ovulatory sizes when exposed to a significant increase in LH pulse frequency (chapter 4). In another experiment presented in chapter 4 of this thesis, suppression of the frequency of secretion of pulses of LH, in intact cyclic ewes, to less than half of the frequency of the luteal phase of the estrous cycle, did not hinder normal follicular wave emergence and growth. *From the results reported in this thesis (chapter 4) and from the work of others, it appears that only very low serum concentrations of LH, with very few pulses, are required to support ovarian follicular wave emergence and growth in the cyclic ewe.*

In anestrous ewes, supra-physiological serum concentrations of estradiol alone or in combination with physiological serum concentrations of progesterone, created by steroid-releasing implants, abolished LH pulses (chapters 3 and 5). In cyclic ewes (Barrett et al., 2006), when similar sized estradiol implants were used, supra-physiological concentrations of estradiol had no significant effect on the LH secretory pattern. However, in cyclic ewes, estradiol implants producing higher concentrations of estradiol did abolish pulsed LH secretion (Barrett et al., 2007). Therefore, the findings above confirm that estradiol exerts a stronger negative feedback effect on LH secretion during seasonal anestrous compared to the breeding season (Goodman et al., 1981a; Joseph et al., 1992; Karsch et al., 1980; Martin et al., 1983). *In other words, we could say that there is a higher threshold for suppression of LH secretory pulses by estradiol-17ß in cyclic ewes compared to anestrous ewes (Goodman and Karsch, 1980; Joseph et al., 1992; Martin et al., 1983).*

It is intriguing that serum estradiol concentrations were not affected by immunization against GnRH in anestrous ewes (chapter 6). In immunized ewes, we expected a decrease in estradiol concentrations as there was a decrease in serum LH concentrations and an absence of pulsatile LH secretion after the booster immunization (Campbell et al., 1997; Campbell et al., 1998). However, ewes produce large follicles that are less estrogenic during anestrous compared to the breeding season (Bartlewski et al., 1998). It was also intriguing to see the gradual increase in estradiol concentrations from scanning period 1 to 3 within both control ewes and ewes immunized against GnRH (chapter 6). This could be due to the effect of season; ewes progressed from mid anestrous to the time of transition to the breeding season (May to mid-July) during the

study. It is believed that large ovarian follicles become more estrogenic in the transition from anestrous to the breeding season (Bartlewski et al., 1999c).

### 8.1.4 Regulation of follicular waves by estradiol and progesterone

In anestrous ewes, treatment with implants releasing only estradiol had no effect on the number of FSH peaks, or peak concentration and amplitude of FSH (chapters 3 and 5). In contrast, in cyclic ewes (Barrett et al., 2006), treatment with similar implants releasing estradiol resulted in truncation of the transient peaks in serum FSH concentrations that precede ovarian follicular waves. Further, treatment of anestrous ewes with implants releasing estradiol and progesterone, suppressed the amplitude of the transient peaks in serum FSH concentrations (chapter 5). *This clearly suggests that progesterone enhances the inhibitory effects of estradiol on FSH secretion leading to a decrease in the amplitude of the peaks in serum FSH concentrations that precede and trigger follicular waves in anestrous ewes.* It is also believed that the inhibitory effects of progesterone are more pronounced in seasonally anestrous ewes compared to ewes in the breeding season (Karsch et al., 1987). Treatment of anestrous ewes with implants releasing only estradiol had no effects on basal serum concentrations of FSH, but mean serum FSH concentrations were suppressed (chapters 3 and 5). Treatment with implants releasing progesterone and implants releasing estradiol; again, had no effect on basal FSH secretion but suppressed mean serum FSH concentrations (chapter 5). *Therefore, the only additive effect of giving progesterone and estradiol to anestrous ewes was to reduce the amplitude of the peaks in serum FSH concentrations that precede follicular waves.*

### 8.1.5 Assessment of markers for follicular quality in follicles from the penultimate and final wave of an estrous cycle

The PGF2a and MPA treatment model described in this thesis was found to increase the ovulation rate but not fecundity in a non-prolific breed of sheep, the Western White Face (Bartlewski et al., 2003). This treatment model resulted in an increase in ovulation rate by causing ovulation of follicles from the penultimate wave in addition to the final wave of the cycle (Bartlewski et al., 2003). However, when ewes were bred after using this treatment, there was no increase in the number of lambs born (Davies, 2005). Follicles growing in the penultimate wave were obviously older at ovulation compared to those from the final follicular wave of the cycle. As there was no increase in the lambing rate following the enhanced ovulation rate caused by treatment with PGF2a and MPA, we hypothesized that the follicles originating from penultimate wave were not as viable as those from the final wave of the cycle. However, in a previous study in sheep, it was found that embryo quality and fertility was not affected by the age of follicles (Evans et al., 2001b). In contrast, in cattle, it was shown that there is early maturation of oocytes in aged follicles leading to a decrease in follicle quality (Austin et al., 1999; Mihm et al., 1999). In the study described in chapter 7 of this thesis, we found that there was a decrease in vascularization or angiogenesis, gap junctional communication and cell proliferation in preovulatory follicles from the penultimate compared to final wave of the cycle following the PGF2a and MPA treatment. *We suggest that when the lifespan of follicles from the penultimate wave of a cycle is extended, they are not as functionally viable as follicles from the final wave of*

*the cycle. It appeared that PGF2a and MPA treatment could be used as an experimental model to study aged follicles.*

## 8.2 Conclusions

1) The length of the estrous cycle does not differ amongst cycles with three or four follicular waves.
2) The mechanism that creates a three-wave or four-wave cycle is unclear as there were no apparent consistent differences in the endocrine or follicular characteristics between three- and four-wave cycles that would suggest a clear regulatory pattern.
3) The inter-wave interval was longer for the first and the ovulatory wave of the cycle in three- compared to four-wave cycles; however, there were no consistent trends or changes in the length of specific phases of a wave that explained this.
4) The length of the growth phase and lifespan of the largest follicle growing in the last or ovulatory wave was longer in cycles with three compared to four waves.
5) Some clear trends for endocrine and follicular characteristics were noted across the cycle in ewes regardless of the number of waves per cycle: a) The length of the lifespan and regression phase of the largest follicle of a wave declined across the cycle as FSH peak concentration and amplitude decreased b) The maximum follicular diameter of the largest follicle growing in the first wave and the ovulatory wave of the cycle was greater compared to other waves of the cycle c) The inter-wave interval was longer for the first wave and the ovulatory wave of the cycle compared to other waves of the cycle.

6) When ewes during the luteal phase of the cycle are exposed to an LH pulse frequency similar to that seen in the follicular phase, follicular waves can grow and function in a similar manner to ovulatory follicles growing in the follicular phase of the cycle.
7) Waves of ovarian antral follicular growth can occur at LH pulse frequencies lower than those seen in the luteal phase of the cycle, but some level of pulsatile LH secretion is required for an FSH peak to trigger emergence of follicular waves in anestrous ewes.
8) Progesterone enhances the inhibitory effects of estradiol on FSH secretion in anestrous ewes, specifically suppressing FSH peak amplitude.
9) FSH peaks continue to occur in the absence of follicular wave development; therefore an endogenous rhythm independent of secretory products from the follicles growing in a wave may drive the rhythmic occurrence of FSH peaks.
10) The major driving stimulus for FSH peaks remains after immunizations against GnRH.
11) Expression of some markers of vascularization/angiogenesis, gap junctional communication and cell proliferation, appeared to be decreased in follicles from the penultimate compared to the final wave of the cycle when the lifespan of the follicles from the penultimate wave was extended. We concluded that such follicles from the penultimate wave of a cycle are not as functionally viable compared to follicles from the final wave.

## 8.3 Practical Implications

Understanding the gonadotropic regulation of ovarian antral follicular wave emergence and growth in cyclic and anestrous ewes will help us to develop effective methods to control reproduction and fertility in the ewe. It will help us to develop more precise methods to induce and synchronize estrus and ovulation for artificial insemination and embryo transfer; and to increase the ovation rate and litter size in non-prolific WWF ewes.

## 8.4 Future Studies

1) As we could not identify any particular mechanism responsible for making three or four wave cycles, comparison of cycles with three and four waves (chapter 2) could be repeated with a greater number of sheep. Additional objectives would be to determine differences in the patterns of LH secretion and inhibin concentrations in serum amongst cycles with three and four waves.
2) In cattle, it was recently shown that there was a two-fold greater chance for repeatability of a particular wave pattern in the estrous cycle, within a herd throughout the year, than for cycles where the wave patterns changed. No such studies have been performed in sheep (Jaiswal et al., 2009). The objective of such a study would be to determine if the wave pattern (three or four waves) is repeatable within a ewe throughout the year.
3) Fertility studies have been performed in cattle that had two or three follicular waves in a cycle. Results were contradictory; some authors reported no differences (Ahmad et al., 1997; Bleach et al., 2004) whereas others reported a decrease in pregnancy rate in cattle with two compared to three waves in a cycle

(Townson et al., 2002). Such studies are lacking in sheep. It would be interesting to breed ewes with three and four waves per cycle and determine whether there are any differences in fertility.

4) The length of the growth phase and lifespan of the largest follicle growing in the ovulatory wave was longer for the three-wave compared to four-wave cycles (chapter 2). We could assume that the preovulatory follicles growing in three wave cycles may contain a relatively aged oocyte compared to four wave cycles. It would be useful to collect preovulatory follicles from the ovulatory wave in three and four wave cycles to assess their quality with respect to the markers of follicular development listed in chapter 7 of this thesis. In addition, the oocytes from these follicles could be aspirated for invitro oocyte maturation and fertilization studies.

5) Recently, ultrasound image attributes of developing ovarian follicles throughout the estrous cycle were studied in sheep (Toosi et al., 2009). It was demonstrated that antral follicles emerging at different stages of the estrous cycle had differences in pixel characteristics of their wall and antrum. It would be interesting to determine whether there are any changes with respect to ultrasound image attributes of follicles growing in cycles with three and four waves.

6) In anestrous ewes treated for 10 days with estradiol releasing implants, pulsed LH secretion was abolished and the emergence of follicular waves was blocked, but the FSH peaks that trigger follicular waves were only affected to a limited extent (chapter 3). In cyclic ewes, extending the length of treatment gave more

profound effects on ovarian follicular growth (Barrett et al., 2007). Using larger size implants (20 cm) or a longer term treatment in anestrous ewes would be an obvious next step, particularly to see the effects on FSH secretory peaks in anestrous ewes.

7) GnRH immunization in anestrous ewes (chapter 6) could be repeated to look at the long term effects of immunization. In addition, the immunized ewes could be treated with ovine LH (oLH) to restore LH pulsatality, while FSH peaks are still present. This would confirm that pulsed LH secretion is required to restore follicular waves in anestrous ewes.

8) The role of FSH peak amplitude in maintenance of the pool of small follicles was not clear based on the results of the present thesis (chapter 5) and work of others (Barrett et al., 2007)). We plan to increase or decrease FSH peak amplitude by treatment with oFSH or steroid releasing implants respectively, in cyclic and anestrous ewes, to further investigate the effects of FSH peaks on maintenance of the small follicle pool.

9) We found that follicles from the penultimate wave are not as functionally viable as follicles from the final wave of the cycle (chapter 7). As a follow up, we plan to investigate oocyte competence and invitro maturation of oocytes of follicles from the penultimate and final waves of the cycle in PGF2a and MAP treated WWF ewes. Further, hormones such as estradiol, progesterone, free IGF, IGFBP and inhibin could be estimated in the follicular fluid from these follicles. We also wish to compare follicles in the penultimate and final wave of the cycle from untreated prolific Finn ewes.

10) As a follow up to our studies in the PGF2a and MAP model, we could assess markers of luteal development and function amongst corpora lutea formed following ovulation of follicles from the penultimate and final waves in WWF ewes. In addition, we could compare these results to those for follicles in the penultimate and final wave from Finn ewes.
11) Microarray analysis could be applied to compare aged follicles from the penultimate wave of the cycle to follicles from the final wave, in Finn ewes and WWF ewes treated with PGF2a and MPA to determine the genes that are differentially expressed; results could be validated using RT-PCR. This gene expression study would allow development of hypotheses to address the differences in follicular quality between aged and normal follicles.
12) Possible changes with respect to ultrasound image attributes of follicles growing in the penultimate (aged) and final wave (normal follicles) of the cycle in PGF and MPA treated WWF ewes and untreated prolific Finn ewes could be studied.
13) Systemic and local effects of the CL have been reported in sheep (Bartlewski et al., 2001; Contreras-Solis et al., 2008). However, the effect of different numbers of CL on follicular dynamics in sheep has not been studied in detail. It would be interesting to study the differences in follicular dynamics and endocrine profiles amongst ewes with single and multiple ovulations.
14) In sheep, it was demonstrated that ovarian antral follicles could be collected at any specific stage of the estrous cycle or follicular wave, to determine the expression of various regulators of follicular development (Duggavathi et al., 2006). We plan to use the same method for follicle collection and assess the

expression of angiogenic/vascularisation factors, markers of gap junctional communication and cell proliferation in follicles collected at different phases of growth (small, medium and large follicles) and regression or preovulatory status, as determined by ultrasonography.

15) Treatment of cyclic ewes with frequent injections of GnRH, during the mid-luteal phase of the cycle, caused development of so called "induced follicular dominance" (chapter 4). We plan to use this experimental model to compare dominant follicles induced during GnRH treatment with normal preovulatory follicles from wave 1 or final or ovulatory wave of the cycle, where dominance is not seen. We could look at concentrations of estradiol, progesterone, free IGF and IGFBP in follicular fluid. In addition, we would assess the expression of aromatase, LH and FSH receptors, IGF-1, IGFBP, VEGF, NOS3, and PCNA in these follicles.

16) In the experimental model above (chapter 4), we assumed that the increased serum concentrations of estradiol caused by the GnRH treatment suppressed FSH secretion and blocked emergence of a follicular wave. It would be interesting to see if follicular wave emergence could be re-initiated with an exogenously created peak in FSH concentrations during the period of induced dominance. Further, we could treat separate groups of ewes with an aromatase inhibitor during the period of induced dominance, to see whether the effect of follicles with induced dominance are blocked and FSH peaks and follicular waves re-established.

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